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CHARACTERIZATION OF Gαq-coupled RECEPTOR SIGNALLING VIA THE NOVEL EFFECTOR PKCζ

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Este trabajo ha sido realizado
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(CSIC-UAM)

Este trabajo ha sido realizado en el Centro de Biología Molecular Severo Ochoa (CSIC-UAM) bajo la dirección de Catalina Ribas Núñez, Profesora Titular del Departamento de Microbiología de la Universidad Autónoma de Madrid y de Federico Mayor Menéndez, Catedrático del Departamento de Biología Molecular de la Universidad Autónoma de Madrid. Además, parte de los resultados fueron obtenidos en la Universidad de Montreal bajo la dirección del Stephen Michnick y en la Universidad de Leicester bajo la dirección de Andrew Tobin.

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A mi abuela,

A mi hermano.

"Un par de horas después, en el mismo cuarto, entre periodistas, fotógrafos y gendarmes, el comisario Treviranus y Lönnrot debatían con serenidad el problema.

- No hay que buscarle tres pies al gato- decía Treviranus, blandiendo un imperioso cigarro-. Todos sabemos que el Tetrarca de Galilea posee los mejores zafiros del mundo. Alguien, para robarlos, habrá penetrado aquí por error. Yarmolinsky se ha levantado; el ladrón ha tenido que matarlo. ¿Qué le parece?

- Posible, pero no interesante- respondió Lönnrot-. Usted replicará que la realidad no tiene la menor obligación de ser interesante.
Yo le explicaré que la realidad puede prescindir de esa obligación,
pero no las hipótesis."

Jorge Luis Borges,
Ficciones, 1956

"It will be objected that the book deals too much with mere appearances, with the surface of things, and fails to engage and reveal the patterns of unifying relationships which form the true underlying reality of existence. Here I must confess that I know nothing whatever about true underlying reality, having never met any. There are many people who say they have, I know, but they´ve been luckier than I am"

Edward Abbey
Desert solitaire, 1968

"Ahora cualquiera sabe que las regiones más valiosas de la realidad (las más valiosas para el hombre y su destino) no pueden ser aprehendidas por los abstractos esquemas de la la lógica y de la ciencia. Y que si con la sola inteligencia no podemos cerciorarnos de que existe el mundo exterior, [...] a menos que neguemos realidad a un amor o a una locura, debemos concluir que el conocimiento de vastos territorios de la realidad está reservado al arte y solamente a él."

Ernesto Sabato,
El escritor y sus fantasmas, 1963

Lo más normal es que abras un libro, te saltes elegantemente el prólogo, la nota del editor y demás morralla, y empieces a leer el primer capítulo. Con las tesis sucede justo lo contrario; nadie pasa de las primeras páginas. Quizás un lector especialmente ocioso hará un recorrido global durante medio minuto inspeccionando formas y colores llamativos. Aún así, probablemente te señale alguna errata que ha pasado inadvertida durante tus cincuenta lecturas de revisión. Pero en general pocos son los aventurados que van más allá. Y no es que los agradecimientos sean una pieza maestra de la literatura (como ya os habréis percatado después de 7 líneas llenas de *nada*) sino que por demérito del resto de las secciones (un auténtico tostón), es la única parte mínimamente leíble en una tesis. [Nota: Si eres un miembro del tribunal debes saber que los resultados de esta tesis son extremadamente novedosos e interesantes y que todo lo dicho anteriormente no son más que figuras retóricas sin fundamento]. Bueno, decía que ésta es la parte más popular de la tesis. Aquí es donde tengo que advertiros, estimados lectores de agradecimientos, que puede que acabéis lamentando no haber pasado directamente a la introducción. Debéis saber que mi silencio ha sido comparado con la tranquilidad que se queda en la cocina después de apagar el extractor de humos. El que avisa no es traidor.

Empezaré a agradecer a los que no agradezco, a todas las personas cuyo nombre no va a aparecer más abajo. Por mi mala cabeza o por falta de lucidez para reconocer a todo aquel que ha contribuido, en mayor o menor medida, a que yo acabe una tesis. Dejémoslo en una especie de homenaje al soldado desconocido.

Sin el apoyo de diversos patrocinadores esta tesis no hubiera podido ser realizada. Gracias al Instituto de Salud Carlos III, principal fuente de financiación, y a las sociedades EMBO, IUBMB, FEBS y SEBB por distintas ayudas de viajes y estancias en el extranjero. Gracias a los congresos, esos lugares imprescindibles para recuperar la ilusión. Gracias al CBM y todo el extraordinario personal siempre dispuesto a ayudar, especialmente los servicios de citometría y microscopía.

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Cuando entré en el laboratorio 320 se llamaba 201. Estaba siempre abarrotado de gente, lleno de papelajos, de tubos y cachivaches diversos. En realidad, desde aquellos tiempos inmemoriales, lo único que ha cambiado en el laboratorio es el número de la puerta. Hacia dentro todo se ha mantenido, y siempre que se ha ido alguien bueno (siempre que se ha ido alguien), ha sido invariablemente reemplazado por otra persona estupenda. Todos sabemos que esto no sucede por casualidad. Esa es la primera razón por la que agradezco a Fede haberme dado la oportunidad de unirme al grupo. Porque el lugar moldea a la persona y yo soy mejor que cuando entré. Decía Groucho Marx aquello de "no deseo pertenecer a ningún club que acepte como socio a alguien como yo". Yo tendría que añadir la salvedad de este laboratorio. Gracias Fede por tu continuo apoyo y disponibilidad para discutir la más ridícula de mis ocurrencias, por tus útiles consejos, por la calidad humana y el sentido del humor. Espero encontrarme más gente como tú en el camino.

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Una vez alguien de mi entorno cuya identidad no revelaré, intentó explicar en qué consiste mi trabajo y después de un rato de reflexión concluyó: Mutilación celular. Porque existe vida más allá de la ciencia, gracias a todos los que no tiene ni idea de lo que hago. Mucho mejor así. A mis compañeros de andanzas segovianos, seguís siendo tan imprescindibles como siempre. Viva el cochinillo y el *buenomajo*. Gracias a Odín, Adri, Miguel, Bea, Violeta, Andrés, Irene, Sofía y Tomaso. A Carlos, por enseñarme a ser valiente, porque tiene un corazón tan grande que los médicos tuvieron que tomar cartas en el asunto. Gracias a Andrea porque ahora, en cuestiones no académicas, es ella la que me deja los apuntes. A Dave, que es de lo mejorcito que salió de esas islas nubladas. A la pandilla de la facultad; Miguel, Cris, Juan, Coca, Jaime, Álex, Belén, Noemí, Lucas y Ana. Gracias a Lígula; Nacho, Eli, Saúl, Borja, Jevi y Carlos, porque hablamos el mismo idioma. Gracias a Carlos Urrutia, el mejor profesor que he tenido nunca. Gracias a Zaira por todo lo que compartimos y a mi familia inglesa, Michael, Annie, Mimi, Zoe y Niki. A Nili. A mi amigo Alberto, y a Beej, aunque estén lejos. A Pablo, compañero de viajes. A Carlos y Mercedes, la pareja de moda.

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ABBREVIATIONS

7TM: Seven transmembrane receptor

AC: Adenilyl cyclase

AKAP: PKA anchoring protein

AngII: Angiotensin II

ANP: Atrial natriuretic peptide

AR: Adrenergic receptor

AT1R: Angiotensin receptor 1

ATP: Adenosine Tri-Phosphate

BAD: Bcl-2-associated death promoter protein

MHC: Myosin heavy chain

MMP: Matrix Metaloproteinase

BRET: Bioluminescence resonance energy transfer

BSA: Bovine serum albumin

BTK: Bruton tirosine kinase

cAMP: Adenosine 3'5' Cyclic Monophosphate

Cdc42: Cell division cycle 42

Ci: Curie

CREB: cAMP Response Element Binding Protein

C-term: Carboxyl terminus

DAG: Diacyl glycerol

dH20: Distilled water

DH: Dbl homology

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

DNA: Desoxyrribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EE: Protein tag with two glutamic acids

EGF: Epithelial growth factor

EGTA: Ethylene glycol tetraacetic acid

FAK: Focal adhesion kinase

FRET: Fluorescence resonance energy transfer

GABA: Gamma-aminobutyric acid

GAP: GTPase activating protein

GDI: GDP dissociation inhibitor

GEF: Guanine nucleotide exchange factor

GFP: Green Fluorescent Protein

GIRKs: Inwardly Rectifying K+ channels

GPCR: G protein coupled receptor

GRK: GPCR kinase

GSK3: Glycogen Synthase Kinase 3

GST: Glutathione S-Transferase

GTP: Guanine triphosphate

GDP: Guanine diphosphate

GLUT4: Glucose transporter 4

h: Hour

HEPES: N 2 Hydroxyethylpiperazine N 2 Ethanesulfonic Acid.

HRP: Horseradish peroxidase

HA: Hemagglutinin

HB-EGF: Heparin Binding Epidermal Growth Factor

IGF-1: Insulin Like Growth Factor 1

IKK: Inhibitor of KappaB Kinase

IL-1: Interleukin-1

IP3: Inositol 1,4,5 trisphosphate (Ins (1,4,5)P3); Inositol 3-phosphate

JAK: Janus family of protein tyrosine kinases

JNK: Jun N-terminal Kinase.

LARG: Leukemia-associated Rho GEF

LB: Luria-Bertani broth

LIF: Leukemia inhibitory factor

MAPK: Mitogen-Activated Protein Kinase

MEF2: Myocyte enhancer factor

MEK: MAPK and extracellular signal-regulated kinase (ERK) kinase

mTOR: Mammalian target of rapamycin

MVBs: Multivesicular bodies

m: Mili

μ: Micro

n: Nano

NFAT: Nuclear Factor of Activated T Cell

NGF: Neural growth factor

NP40: Nonidet P-40

NOX: Nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase

NFκB: Nuclear factor κB

N-term: Amino terminus

p: p value

PAGE: Polyacrylamide gel electrophoresis

Par: Partitioning protein

PBS: Phosphate Buffer Solution

PB1: Phox /Bem 1

PDK1: Phosphoinositide-dependent protein kinase 1

PH: Pleckistrin homology

PIP2: Phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2)

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein kinase A

PKCζ: Protein kinase C zeta

PLC: Phospholipase C

PMSF: Phenylmethylsulfonyl fluoride

PS: PKCζ pseudosubstrate myristoylated inhibitory peptide

PTX: Pertussis toxin

PVDF: Polyvinylidene fluoride

RACK: Receptor for activated C-kinase

RGS: Regulator of G protein signalling

RH: RGS homology

RNA: Ribonucleic acid

rpm: Revolutions per minute

ROCK: Rho-kinase

ROS: Reactive oxygen species

RSK: Ribosomal protein S6 kinase

SEM: Standard error of the mean

SD: Standard deviation

SDS: Sodium Dodecyl Sulphate

siRNA: Small interfering RNA

SAPK: Stress activated protein kinase

SDS: Sodium dodecyl (lauryl) sulfate

SOC: Super Optimal broth with catabolite repression

STAT: Signal Transducer and Activator of Transcription

STI: Soybean trypsin inhibitor

t: Time

TEMED: Tetramethylethylenediamine

TNF: Tumour necrosis factor

TGF: Transforming growth factor

U: Units

V: volt

v: Volume

YFP: Yellow fluorescent protein

I. INTRODUCTION

1. G PROTEIN COUPLED RECEPTOR SIGNALLING

Many hormones, neurotransmitters and different stimuli with a paramount role in health and disease elicit specific cellular responses through cell surface receptors. Of the several families of membrane receptors, by far the largest, most versatile and most ubiquitous is that of the seven-transmembrane (7TM) receptors (also referred to as seven-membrane-spanning receptors and G-protein-coupled receptors (GPCRs))(Pierce et al., 2002) (Fig. I1). These receptors are a physical conduit for the transmission of chemical signals across the cell membrane and mediate the activation of intracellular G proteins (Hepler & Gilman, 1992).

a. Historical context of G-protein-coupled receptors

The British pharmacologist John Newport Langley and his student Henry Dale, working together during the first decade of the 20th century, were the first scientists who explicitly stated the idea of a 'receptive substance' on reactive cells (Langley, 1901). Their deductions were based on classical physiological and pharmacological experiments using skeletal or smooth muscle preparations, or submandibular salivary glands, and combinations of adrenoceptor or acetylcholine receptor agonists and antagonists. During the following decades, classical receptor theory was developed from Langley´s contribution into basic pharmacology notions such as affinity and efficacy in drug action (Furchgott, 1964). The 1960s and 1970s witnessed the beginning of a fruitful merge between biochemistry and pharmacology with prominent figures like Sutherland, Krebs and Rodbell, who focused their attention on the molecular basis of hormone and drug action. This led to a series of seminal discoveries that were to shape all further work in the field of cell

signalling. Rall and Sutherland revealed that hormones such as epinephrine and glucagon interact with receptors in liver cell membranes to elicit the production of the second messenger cyclic-3´,5´-monophosphate (cAMP) by adenylyl cyclase (Rall & Sutherland, 1958). Later, Krebs discovered the cAMP-dependent protein kinase, which is the primary effector of cAMP action (Walsh et al., 1968). In 1971, Rodbell proposed the existence of a guanine nucleotide regulatory protein that serves as a transducer between hormone receptors and adenylyl cyclase (Rodbell et al., 1971). Gilman and colleagues subsequently demonstrated the existence of this protein (Ross & Gilman, 1977), which was later purified and named Gs (Gilman, 1987). Retrospectively, these discoveries shed light on the first molecular events that convey signalling from the outside to the inside of the cell.

Still in the 1970s the idea of a physical receptor was very controversial. Even Sutherland, who was confident with the notion that receptors were physicochemical entities, manifested his doubts that they would be independent elements, distinct from adenylyl cyclases, which his own group had discovered (Lefkowitz, 2004). However, shortly after, the development of radiolabelling techniques allowed the first receptors to be identified. Chen-Yuan Lee purified and implemented the use of α-bungarotoxin, an extremely potent, pseudo-irreversible snake toxin that acts selectively on the nicotinic acetylcholine receptor (Changeux et al., 1970). Using 125I-labeled α-bungarotoxin, during 1970 and 1971, three groups identified binding sites that reflected the nicotinic receptor in the electric organ of the electric eel (Snyder & Pasternak, 2003). Subsequently, ligand labelling techniques allowed new insights into the properties of receptors. The coupling of receptors to G proteins in a ternary complex model (Ligand/Receptor/Gprotein) as well as the two different receptor states (high affinity, G protein-coupled and low, uncoupled) were established (De Lean et al., 1980). During the last half of the 1970´s and in the 1980´s several adrenergic receptors were purified in Robert Lefkowitz's laboratory, where the GPCR field was initiated. These adrenergic receptors, that turned out to be single polypeptide chains, were successfully reconstituted in vesicles along with Gs and adenylyl cyclase and this system proved sufficient for catecholamines to catalytically activate the enzyme (Lefkowitz, 2004). In 1986, Brian Kobilka managed to clone the gene and the cDNA of the hamster´s β2 adrenoceptor through genetic library screening (Kobilka et al., 1987). Surprisingly, the gene did not have any introns, a feature later found for other many other GPCRs such as the α1-adrenergic, muscarinic or dopamine receptors. Also, unanticipatedly, the β2-adrenoceptor shared sequence homology and a predicted 7TM architecture with the visual pigment rhodopsin, also coupled to G proteins. This was a key finding leading to the notion of the existence of a GPCR family. Since then, a growing number of receptors were cloned and identified based on suspected or serendipitous homology with already-cloned members of the family. Today GPCRs form a vast –the largest- family of receptors with

over 800 genes that include receptors for many hormones, neurotransmitters, chemokines and calcium ions, as well as sensory receptors for various odorants, bitter and sweet taste and even photons of light. Not only do GPCR regulate many physiological processes, but drugs that target these receptors — either directly or indirectly — account for most of the medicines sold worldwide. However, to date, the functional identity and endogenous ligands of many of the receptors in this family remains unknown, and these are referred to as *orphan receptors*.

Figure I1. G protein coupled receptors (GPCR). New Scientist cover (15/01/2005) portraying GPCR as The Magnificent Seven. Image: Karina Åberg Sakmar, sakmark@me.com.

b. Functional and structural features of GPCR

Despite the remarkable chemical diversity of the activating ligands and the clear differences in receptor amino acid sequences, all GPCRs are predicted to share a common three-dimensional fold consisting of seven transmembrane helices linked by alternating intracellular and extracellular loops. The many proteins that belong to the GPCR superfamily can in turn be grouped into three different families (A, B and C) on the basis of sequence similarity. Sequences within each family generally share over 25% sequence identity in the transmembrane core region, and a distinctive set of highly conserved residues and motifs. Among the three families, little similarity is evident beyond the predicted 7TM architecture (Pierce et al., 2002). Family A is the largest group and includes, amongst others, the receptors for light (rhodopsin), adrenaline (adrenergic receptors) or odours. Family B contains only 25 members, all coupled to Gs and including the receptors for the

gastrointestinal peptide hormone family like glucagon, corticotropin-releasing hormone, calcitonin and parathyroid hormone. Family C is also relatively small, and comprises receptors with a very large extracellular amino terminus including the metabotropic glutamate receptor family, the GABAB receptor, and the calcium-sensing receptor, as well as some taste receptors.

GPCRs bind heterotrimeric Gαβγ proteins, their intracellular partners and the first elements of the cascade initiated upon activation of the receptor. Agonist binding to the receptor seems to involve rearrangements of helices 6 and 3. A recent structure of the GPCR rhodopsin in a constitutively active state and comparison with the structure of ground-state rhodopsin suggests that rotation of transmembrane helix 6 is the critical conformational change in activation (Standfuss et al., 2011). These was further confirmed by the crystal structure of an activated β1 adrenergic receptor in complex with Gαi (Rasmussen et al., 2011). Activated receptors can interact with the heterotrimeric G protein and act as a GEF (guanine nucleotide exchange factor) promoting GDP dissociation, and GTP binding and activation. The activated heterotrimer dissociates into a GTP-bound α subunit and a βγ dimer, both of which have an independent capacity to regulate an ever-expanding list of independent effectors. Hydrolysis of GTP to GDP, a process known to be modulated by RGS (regulator of G-protein signalling) proteins, causes re-association of the heterotrimer and termination of the activation cycle. However, a report has challenged the current model of a physical dissociation of α from βγ upon activation, since it was shown that non dissociable heterotrimeric G proteins could also initiate signalling (Klein et al., 2000) .

GPCRs have traditionally been considered monomeric membrane proteins but several lines of evidence obtained during the last two decades suggest that GPCRs can also form homo- and heterodimers. Assembly of different types of GPCRs into dimers would then create receptor complexes with unique biochemical and functional characteristics (Pin et al., 2007). However, the existence of such receptor dimers in native tissues remains uncertain because of the difficulties in identifying GPCR complexes in a native environment. Most data showing the occurrence of dimers comes from energy transfer experiments between two receptors [namely, fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET)] but there is discussion over the question of how close two receptors should be to be considered a dimer (Lohse, 2010).

c. Heterotrimeric G proteins

Upon activation heterotrimeric G proteins are dissociated into the monomeric alpha subunit and beta/gamma dimeric complex due to the exchange of GDP for GTP in the nucleotide binding pocket of Galpha. GTP-bound Gα proteins are then able to interact with

different effectors thereby initiating an extensive number of cellular functions (Fig. I2). Besides, free βγ dimeric complex also binds a plethora of effectors leading to the activation of alternative pathways.

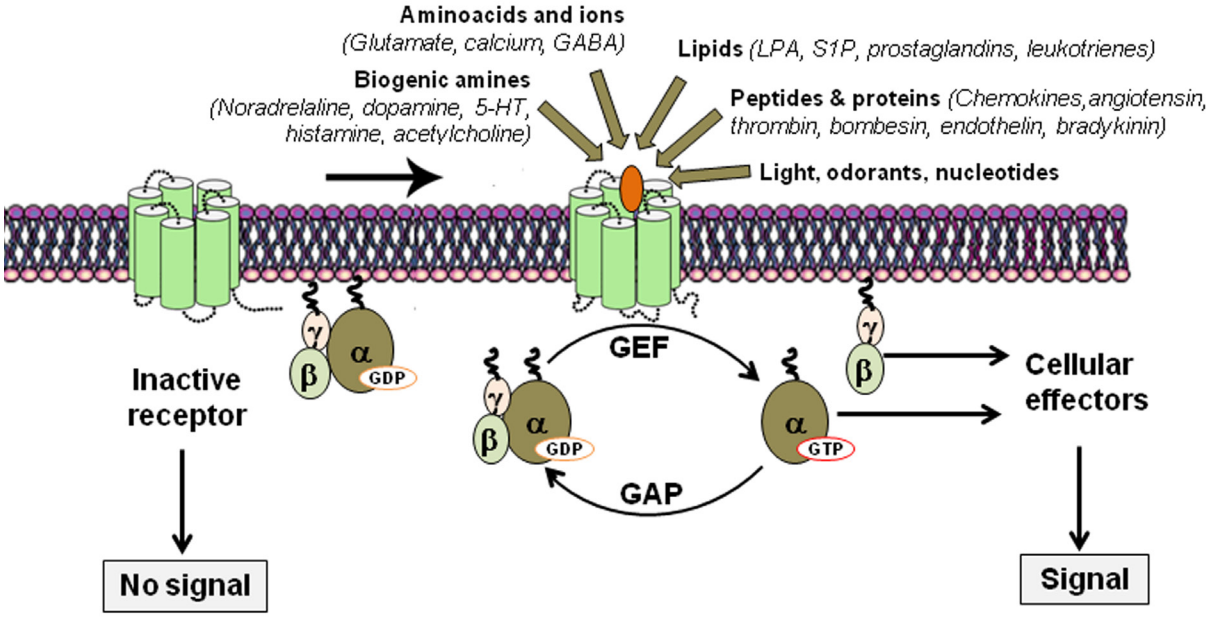

Figure I2. GPCR activation cycle. A huge diversity of ligands bind and activate GPCRs. In turn, receptors activate heterotrimeric G proteins by promoting GDP to GTP exchange in the Gα subunit and dissociation from the βγ subunit. Both Gα and βγ initiate signalling through different effectors.

Despite the size and diversity of the GPCR superfamily, there are a relatively small number of G proteins to initiate a large number of different intracellular signalling cascades. In the human genome 35 genes encode G proteins, 16 of which correspond to α-subunits, five to β and 14 to γ (Milligan & Kostenis, 2006). On the basis of sequence similarity, the Gα subunits have been divided into five different families (Gs, Gi, Gαq, G12(Simon et al., 1991) and the newly discovered Gv (Oka et al., 2009)) and this classification has served to define both receptor and effector coupling. Since the signal transducing properties of the various possible βγ combinations do not appear to radically differ from one another, these classes are defined according to the isoform of their α subunit (Wettschureck & Offermanns, 2005).

While most GPCRs are able to activate more than one Gα subtype, often they also show a clear preference for one subtype over another (Gudermann et al., 1997). The functional selectivity of the receptor towards a certain subtype of Galpha is determined by the specific conformation of the GPCR´s GEF domain that is stabilised by the ligand. For instance, β adrenergic receptors couple to Gs, muscarinic M1/M3/M5 receptors couple to Gαq/11 whereas muscarinic M2/M4 receptors couple to Gi. However, more than one

conformation can be stabilised which results in the activation of more than one subtype. Notable examples of this are G12/13-cupled GPCRs that are also capable of coupling to other G proteins, often Gαq/11. Also, if the availability of the preferred subtype is low, the activation of other isoform can occur and, also, receptor phosphorylation in internal loops due to feedback loops can result in G protein preference switch.

The Gs-initiated cascade was the first cell signalling pathway to be described, and many key concepts, including that of second messenger, protein phosphorylation and signal transducers arouse from its characterization (Neves et al., 2002). The Gs family together with the Gαi/o family share a same effector, the adenylyl cyclase (AC). While there are ten different AC gene products in mammals, each with subtle differences in tissue distribution and/or function, all catalyze the conversion of ATP into the second messenger cAMP, and all are directly stimulated by G-proteins of the Gαs class. Conversely, the interaction of Gαi/o and AC is inhibitory thus preventing the enzyme from generating cAMP. The level of cytosolic cAMP may then determine the activity of various ion channels, the Rap GEFS termed EPACs, as well as members of the Protein Kinase A (PKA) family (Milligan & Kostenis, 2006). The Ga12/13 subclass activates three RhoGEFs (p115-RhoGEF, PDZ-RhoGEF, and LARG), that are responsible for the activation of the small GTPase Rho. Once bound to GTP, Rho can interact with a myriad of proteins responsible for cytoskeleton regulation such as the Rho-kinase (ROCK) (Milligan & Kostenis, 2006). The effectors of the Gαq/11 pathway are the main subject of this introduction and will be covered in detail over the next section. No functional data has yet been gathered for the recently discovered Gv class.

Gβγ subunits are also capable of binding to and activating a number of cellular proteins with a particular relevance in Gαi/o-coupled GPCR signalling. The primary effectors of Gβγ are various ion channels, such as G-protein-regulated Inwardly Rectifying K+ channels (GIRKs), P/Q- and N-type voltage-gated Ca2+ channels, as well as some isoforms of AC, PLCβ and Phosphoinositide-3-Kinase (PI3K) (Milligan & Kostenis, 2006).

d. GPCR regulation

In addition to the GTPase activity of Gα subunits, signalling via G protein-coupled receptors (GPCR) is classically terminated at the receptor level by a two-step mechanism: a GPCR kinase (GRK) phosphorylates the active receptor, converting it into a target for high affinity binding of arrestins. Bound arrestins shield the cytoplasmatic surface of the receptor, precluding G protein binding and activation (Wilden, 1995). As a result of arrestin recruitment, phosphorylated receptors are also targeted for clathrin-mediated endocytosis, a process that serves to resensitise and recycle receptors back to the plasma membrane. An additional regulatory mechanism involves a negative feedback loop from second messenger-activated kinases (PKA or PKC) that phosphorylate GPCRs, thus uncoupling them

from G proteins. In contrast to GRK-mediated phosphorylation, there is no evidence that these kinases promote arrestin binding to the receptor. Alternatively, there are reports showing the occurrence of phosphorylation-independent events of GPCR desensitisation that can be mediated by recruitment of GRKs and also RGS proteins (Ferguson, 2007).

Mammals express seven GRKs (GRK1–GRK7) and four arrestin isoforms, of which GRK1, GRK7, arrestin1 and arrestin4 are only found in the visual system. Of the remaining GRK and arrestin proteins, GRK2, GRK3, GRK5, GRK6, arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2) are ubiquitously expressed (Magalhaes et al., 2012). GRKs are comprised of three functional domains: an amino-terminal regulator of G-protein signalling (RGS) homology (RH) domain, a central catalytic domain and a carboxyl-terminal membrane-targeting domain. GRKs are targeted towards the plasma membrane to phosphorylate serine and threonine residues within the third intracellular loop and carboxyl-terminal tail domains of activated receptors. Both GRK2 and GRK3 encode pleckstrin homology domains that allow association with the Gβγ subunit as well as with phosphatidylinositol 4,5-bisphosphate. Also, their RH domains, but not that of other GRKs, have been involved in Gαq binding and sequestering (Carman et al., 1999b). Indeed, GRK2/3 display an additional level of negative regulation on GPCR signalling; besides phosphorylation and desensitisation of the receptor it also directly binds Gαq and displaces it from receptor and effector binding complexes (discussed in section 5i).

As mentioned above, GRK-mediated phosphorylation promotes the binding of β-arrestins, which function as endocytic adaptor proteins that facilitate the targeting of receptors for clathrin-mediated endocytosis (Ribas et al., 2007) (Fig. I3). Both β-arrestin1 and β-arrestin2 specifically bind to the clathrin heavy chain and to b2-adaptin subunit of the clathrin adaptor protein complex AP2 to facilitate endocytosis (Goodman et al., 1996). In addition, Src-mediated phosphorylation of b2-adaptin regulates the dissociation of the b2-adaptin/β-arrestin complex (Zimmerman et al., 2009). Thus, clathrin-coated pits invaginate inwards and pinch off to form a clathrin-coated vesicle in a process that requires the GTPase dynamin. Several clathrin-independent pathways of endocytosis also exist, involving lipid rafts and caveolin-enriched domains, although the precise mechanisms and structural components involved in these pathways are not well understood. Internalised receptors, as any other endocytic cargo, follow two different endocytic routes leading to either degradation or dephosphorylation and recycling back to the plasma membrane. Endosomal trafficking is controlled by several small GTP-binding proteins of the Ras superfamily named Rab proteins (Fig. I3). Following GPCR internalization into early RAB5-containing endosomes, they can rapidly recycle back to the plasma membrane by a RAB4-mediated process, traffic to the recycling compartment that contains RAB11A or remain in endosomes, which mature into multivesicular bodies (MVBs) and late endosomes. Early-to-late endosome maturation involves the acquisition of RAB7 and the removal

of recycling endosomal components. Fusion of late endosomes and MVBs with lysosomes carrying proteolytic enzymes results in cargo degradation (Sorkin & Von Zastrow, 2009). Also, receptor ubiquitylation serves as a chlatrin-coated pit targeting signal and plays a key role in lysosomal targeting (Hislop & Von Zastrow, 2010). β-arrestins bind MDM2, an E3 ubiquitin ligase best known for its role in regulating the tumor suppressor p53. When a receptor such as β2AR or the vasopressin receptor is stimulated, it binds β-arrestin, triggering MDM2-mediated ubiquitination of the β-arrestin (Shenoy et al., 2001). This ubiquitination step is necessary for β-arrestin to perform its adaptor role in clathrin-mediated endocytosis, although the mechanisms remain to be elucidated.

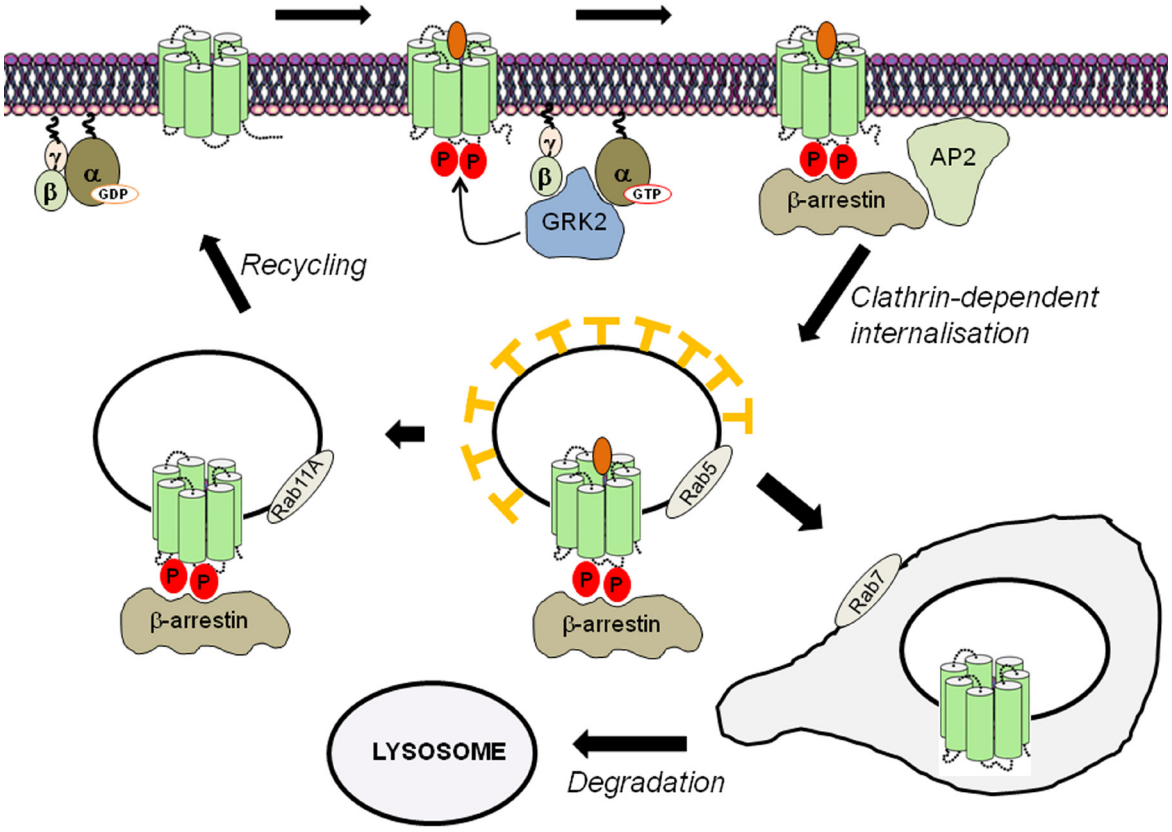

Figure I3. GPCR deactivation cycle. Activated GPCR is phosphorylated by GRKs on the internal loops. Phosphorylated residues are recognised by arrestins that, together with AP2, promote clathrin-mediated receptor internalisation into Rab5-containing vesicles. These can progress into Rab7-containing multivesicular bodies and degradation, or into Rab11A-containing vesicles to recycle back to the plasma membrane.

Endocytosis is an important cellular mechanism to attenuate signal strength and duration. The reduction of physically available receptors for ligand binding leads to a rapid decay in signal and allows for subsequent inputs to be rapidly processed. Failure to undergo desensitisation and the resulting continuous signalling is found in many types

of cancer (Lappano & Maggiolini, 2011). However, mounting evidence supports the occurrence of sustained signalling in endosomes, which act as important signalling platforms. It is proposed that the type of signalling displayed at the endosomes can either be equivalent to plasma membrane signalling, or interestingly, it can also be exclusive for these compartments. In this novel type of signalling, β-arrestin as a multidomain scaffold protein that can bind an ever-expanding array of signalling molecules, plays a key role in the initiation of many different pathways both at the plasma membrane and within the endosomes.

e. Relative contribution of G proteins and β-arrestins in GPCR signalling

The classical model for GPCR action involves G protein-mediated signal transduction and desensitisation by GRKs/β-arrestins. For decades, this understanding has disregarded β-arrestins as mere regulators instead of active players in GPCRs signalling. Over the last few years there has been a new appreciation of the capacity of β-arrestins to act as multifunctional adaptor proteins that have the ability to signal through multiple mediators such as mitogen-activated protein kinases (MAPKs), Src, nuclear factor-κB (NF-κB) and phosphoinositide 3-kinase (PI3K). The action of G proteins and β-arrestin is diverse and are often spatially and temporarily segregated. For instance, the action of G protein/β-arrestin towards the activation of ERK1/2 by the angiotensin II receptor AT1 is additive but differs in kinetics, timeframe and location (Ahn et al., 2004). Upon stimulation of the AT1A receptor, ERK1/2 is immediately, but transiently, activated via the G protein-dependent pathway, whereas β-arrestin2-mediated ERK1/2 activation is relatively slow but persistent. Interestingly, G protein-activated ERK is found in the nucleus whereas it localises in the cytoplasm upon β-arrestin activation. This localisation difference opens new avenues towards the characterization of differential substrates, especially in the cytoplasm where ERK targets have been much less scrutinised. Additionally, mutational studies have determined the independence of both pathways. Mutant versions of the angiotensin AT1A and β2A receptor are unable to couple to G proteins while still correctly recruiting β-arrestins (DeWire et al., 2007).

Upon the characterization of β-arrestins as important signalling nodes, the action of the most known GPCRs is now being revisited in an attempt to characterise the relative contribution of G protein versus β-arrestin for a given response. It seems that the activation strength of one or the other pathway is not always balanced and depends on the stimulus, receptor and cell type. This phenomenon has been referred to as “biased agonism” to describe the preference of a certain ligand (biased ligand) or receptor (biased receptor) towards one of the aforementioned routes. A growing number of examples of

biased agonism have been described in the last few years and it is being actively studied to improve the efficacy of pharmacological therapies. To date, β-arrestin-biased agonists have been chemically developed for the angiotensin AT1A, β1 adrenergic and vasopressin 2 receptors (Rajagopal et al., 2010). In all cases, the impaired ability to activate G protein does not affect β-arrestin mediated responses. In a naturally occurring example of biased agonism, the chemokine receptor CCR7 binds to two different ligands. One ligand, termed ELC/CCL19, can activate both G protein and β-arrestin signalling, and another ligand (SLC/CCL21) activates only G protein signalling and does not recruit β-arrestins (Kohout et al., 2004). However, in this system the independence of each response is not as clear as with synthetic ligands since β-arrestin activity depends upon G protein activation.

It is also noteworthy that the resulting functional and physiological effects of these pathways are usually different and, occasionally, they can antagonise each other. This is especially well illustrated in the heart, where Gαq is a known initiator of cardio-damaging cascades that result in hypertrophy and heart failure (see section 6) and β-arrestin seems to start cardioprotective responses (Noma et al., 2007). Mechanical stress can activate the angiotensin AT1 receptor without the need of its natural ligand through a β-arrestin-biased mechanism. Rakesh et al (2010) reported that mechanical stretch selectively activates the ERK1/2 pathway through β-arrestin, with no signs of G protein activation. This stimulus also led to receptor phosphorylation and internalisation as well as to EGFR transactivation and Akt phosphorylation (Rakesh et al., 2010). These effects mimic those produced by the chemically synthesised AT1R biased agonist, SII, and pose a novel mechanism by which the heart responds to acute challenges by activating survival pathways in a β-arrestin-biased manner. The effect of biased agonists might be a promising therapeutic tool for treating cardiovascular disease. Recently, an angiotensinII agonist that competitively antagonises with G protein signalling, but promotes β-arrestin recruitment and ERK phosphorylation, has been shown to reduce arterial pressure, increase contractility and improve cardiac performance (Violin et al., 2010).

2. THE Gαq/11 FAMILY

The Gαq family of G proteins were initially identified through affinity purification and molecular cloning strategies (Hubbard & Hepler, 2006). There are four family members; Gαq and Gα11 are ubiquitously expressed, Gα14 is found in kidney, liver and lung, and Gα15/16 (mouse/human orthologues, respectively) is only expressed in hematopoietic cells. Gαq is the most widely studied member of the family and will be the main focus of this introduction.

Gαq has been shown to canonically activate the β-isoforms of phospholipase C (PLCβ), their major downstream effectors (Rhee, 2001). Subsequently, membrane-bound PIP2 is hydrolized into diacylglycerol and inositol-1,4,5-triphosphate leading to the activation of conventional and novel protein kinase C (PKC) isoforms and to the mobilization of internal Ca^{2+}, respectively. However, to date Gαq is known to interact with more than 20 proteins and the real complexity of Gαq pathways, driven by interactions between activated G protein and different effectors, is yet very much at its infancy. A crucial study performed by Golebiewska et al (2006) revealed the existence of distinct preassembled Gαq signalling complexes and showed independent binding of Gαq to two different effectors (PLCβ and PI3K) in the same cellular scenario. This was in agreement with the finding that PLCβ cannot account for all of Gαq-mediated cellular functions since the estimated Gαq molecules expressed in a cell significantly outnumbers those of PLCβ (Golebiewska & Scarlata, 2008). Thus, a growing body of evidence suggests alternative Gαq effectors that are responsible for some PLCβ-independent Gαq functions. Such is the case of p63RhoGEF (Lutz et al., 2007), that directly binds to Gαq/11 linking GPCRs and RhoA activation. There also seems to be a competitive relationship between PLCβ and p63RhoGEF for binding to Gαq which suggests the existence of alternative and mutually exclusive Gαq-initiated pathways (Lutz et al., 2005). Most recently we have described a novel signalling axis for the activation of ERK5 by Gq-coupled GPCRs that is independent of the activation of PLCβ. This pathway, which relies upon the interaction between Gαq and two novel effectors (PKCζ and MEK5), will be covered in detail in section 5, together with all known Gαq-dependent signalling.

The Gα subunit structures reveal a conserved protein fold comprising a GTPase domain and α helical domain (Oldham & Hamm, 2008). The GTPase domain is similar to that of other GTP-binding proteins, including monomeric small GTPases and translational elongation factors (Mizuno & Itoh, 2009). The GTPase domain of Gαq participates in the hydrolysis of GTP to GDP and is involved in binding the Gβγ subunit, GPCRs and effectors. Amongst the most highly conserved sequences of this domain are five loops containing the consensus sequences for guanine nucleotide binding and also three flexible loops (termed switches I, II and III) that undergo important conformational changes between the GDP/GTP-bound forms of the protein. The helical domain is composed of six α-helices that form a lid over the nucleotide-binding site. This domain is involved in increasing the affinity of Gα for guanine nucleotides and increasing intrinsic GTP hydrolysis activity of the protein (Echeverria et al., 2000). Since this is the most dissimilar domain amongst the Gα families it might also play certain role in effector specificity.

Not much structural information on the two remaining regions of Gα subunits, the N- and C-terminus, could be gathered from crystal structures as they were disordered

or removed. Both termini are essential for receptor specificity, interaction with the Gβγ subunit and for membrane targeting of the G protein. All Gα subunits studied to date are lipid-modified at their N-terminus, and all except Gαt contain palmitate, a post translationally characteristic of transmembrane and peripheral membrane proteins. Gαq is dually palmitoylated at adjacent cysteines (C9 and C10) (Linder et al., 1993). Fatty acid modification mediates membrane attachment and targeting, promotes interactions with proteins and lipids, and even modulates enzymatic activity of target proteins. Consistently, some studies have shown that receptor stimulation promotes palmitate turnover on Gαq (Stanislaus et al., 1997). Both the palmitoylation and the interaction with the Gαq subunit, which provides some lipid itself (Gγ is prenylated), are key determinants for membrane localisation of the G protein (Wedegaertner et al., 1993; Wise et al., 1997).

a. Structural determinants for Gαq-effector interactions

The effector recognition site in Gα proteins is formed almost entirely of the region extending from the C-terminal half of α2 (Switch II) together with the α3 helix and its junction with the β5 strand (Sprang et al., 2007). Since the structure of these regions is well conserved amongst the different Gα families it is assumed that effector specificity is mostly determined by the aminoacid sequence (Oldham & E. Hamm, 2006). Indeed, as revealed by extensive mutagenesis analysis and crystalised structures, within the same Gα family, different subsets of residues are known to activate different effectors. This implies that although effectors are thought to be mutually exclusive and to compete for binding to the effector binding site (Carman et al., 1999b; Lutz et al., 2005), the specific determinants of these interactions are different.

In all crystalised structures of G protein-effector complexes, a general binding domain within the effectors has been identified. A hydrophobic side chain of the effector would be inserted into the N-terminus of the α2 (switch II) and α3 helices within the G protein. Besides, effector specificity within the same Ga protein is achieved through additional contacts with the α2/β4 and α3/β5 loops. Specifically, available crystal structures of Gαq in complex with three different partners (PLCβ3 (Waldo et al., 2010a), p63RhoGEF (Lutz et al., 2007) and GRK2 (Tesmer et al., 2005)) shed light on the effector-Gαq binding surfaces. Comparison of the structures for PLCβ and p63RhoGEF reveals that both effectors independently evolved a Gαq-binding interface with essentially identical residues. This Gαq-binding surface comprises a helix-turn-helix grafted onto the end of a DH/PH cassette to bind the α3/Sw2 declivity of Gαq (Waldo et al., 2010a). The aminoacid sequence and the helix-turn-helix motif are not conserved in the rest of the reported effectors for other G proteins, except for the hydrophobic character of the region (Ross, 2011). Also,

the regulator GRK2 forms an effector-like interaction with Gαq in a very similar fashion to PLCβ3 and p63RhoGEF, and different to the regulators of G protein signalling (RGS) proteins (Tesmer et al., 2005). Indeed, RGS proteins have been shown to bind into high order G protein-effector complexes without competing for the effector binding site, but instead allosterically inhibiting effector association (Shankaranarayanan et al., 2008) (see section 2c).

b. Effector activation mechanisms

The mechanisms for effector activation by Gα proteins are diverse. For instance, Gαs acts as both allosteric activator and inhibitor of adenylyl cyclase, and Gαi is an allosteric inhibitor. In the activation of GMP phosphodiesterase by Gαt, the G protein sequesters an inhibitory subunit of the effector (Sprang et al., 2007). As for Gαq, the effector-activation mechanism reported for PLCβ and p63rhoGEF seems to be similar. The activity of both proteins is promoted upon Gαq binding by the relief of an autoinhibitory loop buried within an active region of the effector and, in turn, this domain also participates in the activation of the G protein (Lyon et al., 2011; Shankaranarayanan et al., 2010). In the case of RhoGEFs, they contain a catalytic Dbl homology (DH) domain that is immediately followed by a pleckstrin homology (PH) domain (Rossman et al., 2005). In an inactive state, the PH domain inhibits the intrinsic GEF activity of the DHdomain (Welch et al., 2002) and this constrain is released by interactions with the plasma membrane and activator proteins like Gαq. Similarly, the active site of PLC-β3 is occluded by a poorly conserved loop named X/Y linker which is presumably forced out by the surface of the plasma membrane coupled to the engagement of Gαq (Waldo et al., 2010a). Despite membrane targeting is believed to be an important component for the activation of some effectors, especially in the case of RhoGEFs, both PLCβ and p63rhoGEF reside basally at the plasma membrane, so the activation mechanism is proposed to be simple allosterism.

c. Structural basis for Gαq deactivation

Gαq can be deactivated by the enhancement of its intrinsic GTPase activity by RGS proteins (GTPase-activating proteins (GAPs)) as well as effectors such as PLCβ.

The structure of Gαi in complex with RGS reveals that these proteins exert negative regulatory action by stabilising the transition state for GTP hydrolysis (Tesmer et al., 1997). This structure has shown that the interaction footprints in G protein structure are primarily located around the switch I/II, from aminoacids 180 to 220, a region that is supposed not to involve effector binding. Additionally, the structure of Gαq/PLCβ3 has contributed to map out the binding region for GAPs specifically in Gαq. The mechanism

of the interaction, referred to as kinetic sacaffolding (Waldo et al., 2010a), involves rapid activation of PLCβ which, in parallel, deactivates the G protein by promoting GTP hydrolysis. This ancient mechanism provides a structural basis for rapid and transient activation of the G protein. Indeed it was found that both PLCβ and RGS proteins action on Gαq is very similar. Thus, the Gαq surface involved in engaging the GAP function of PLCβ, mostly the switch I and the N terminal part of the switch II, is similar to the reported contacts for RGS proteins (Ross, 2011). Although there is no sequence similarity between PLCβ and RGS proteins, they both carry flexible loops that place asparagines to stabilize residues in Gα that are important for GTP hydrolysis (Ross, 2011). These residues, glutamine 209 and arginine 183, if mutated lead to constitutive activation of the G protein that is unable to hydrolise GTP. Mutation on Gln209 has been recurrently reported as a frequent somatic mutation in uveal melanoma and blue navi (Raamsdonk et al., 2008).

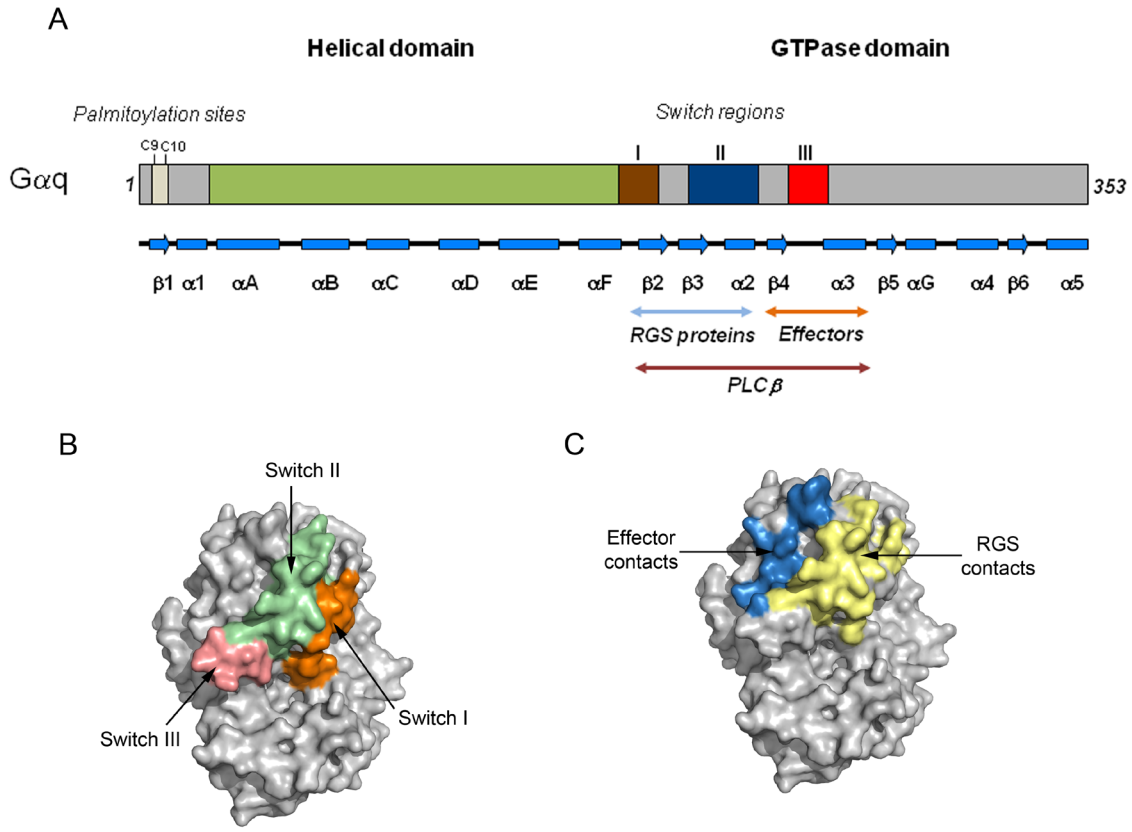

Figure I4. Gαq structure. A) Linear representation of Gαq sequence with corresponding three-dimensional motifs and binding regions. B, C) Crystal structure of Gαq (PDB: 2BCJ) highlighting the conformational change-susceptible switch regions (B) or the effectors/RGS proteins contacts.

3. PROTEIN KINASE C

a. General features

The protein kinase C (PKC) was initially identified as a family of serine/threonine kinases that transduce the myriad of signals promoting lipid hydrolysis. The importance of this protein family in signalling was exemplified by the diverse transduction mechanisms that result in the generation of the main protein kinase C's activator, diacylglycerol. Signals initiated from GPRCs, tyrosine kinase receptors, or non-receptor tyrosine kinases can cause diacylglycerol production, either by activation of phospholipase C or phospholipase D to yield phosphatidic acid and then diacylglycerol (Newton, 1995). In addition, fatty acid generation by phospholipase $\alpha 2$ activation was shown to modulate protein kinase C activity. Thus, multiple receptor pathways feeding into several lipid pathways converge into second messenger-induced activation of protein kinase C. Also, phorbol esters, potent tumor promoters, can stimulate PKC activity and phosphatidylserine (PS), an acidic lipid located exclusively on the cytoplasmic face of membranes, is required by all PKC isozymes. In addition, some isozymes require calcium for optimal activity. However, the classical notion for PKC activation was challenged in the light of novel reported family members that did not fulfill most of the premises above.

To date, there are 12 members of the PKC family which are classified on the basis of structural and activation characteristics (Mackay & Twelves, 2007) (Fig. I5). Classical or conventional PKCs (cPKCs; PLCα, PLCβ1, PKXβII and PKCγ), which are calcium-dependent and activated by both PS and DAG; novel PKCs (nPKCs; PKCσ, PKCδ, PKCε, PKCη and PKCθ), which are calcium independent and regulated by DAG and PS; atypical PKCs (aPKCs; PKCζ and PKCλ), which are calcium independent and do not require DAG for activation, although PS can regulate their activity; and the PKN subfamily (PKN1, PKN2, and PKN3) that can also be regulated by PS.

The structure of all PKCs consists of a regulatory domain and a catalytic domain tethered together by a hinge region (Mellor & Parker, 1998) (Fig. I5). The regulatory domain located at the amino-terminus of PKCs contains C1 and C2 domains and a pseudosubstrate region. The C1 domain is present in all of the isoforms of PKC and has a binding site for DAG and phorbol esters. This domain is functional and capable of binding DAG in both conventional and novel isoforms; however, atypical PKCs have a pseudo-C1 domain since it cannot bind DAG or phorbol esters. The C2 domain only displays calcium sensing properties in conventional PKCs although it is also present in novel isoforms. The pseudosubstrate region, which is found in all classes of PKC, is a small sequence of amino acids that mimics a substrate and binds within the substrate-binding cavity in the catalytic do-

main. It acts as an autoinhibitory loop in the inactive/basal state of the kinase since it lack critical serine/threonine phospho-acceptor residues and cannot be phosphorylated. Additionally, the PKN subfamily possess three leucine-zipper-like heptapeptide repeat 1 domains (HR1) at their regulatory region, which bind Rho-GTP and regulate phosphorylation by phosphoinositide-dependent protein kinase 1 (PDK1)(Diaz-Meco & Moscat, 2012).

The catalytic (kinase) domain is highly conserved among the different isoforms, as well as, to a lesser degree, between the catalytic regions of other serine/threonine kinases. The kinase domain is usually phosphorylated and this is supposed to be essential for its function. Conventional and novel PKCs have three phosphorylation sites located on specific motifs (the activation loop, the turn motif, and the hydrophobic motif). The atypical PKCs and PKN are not phosphorylated on the hydrophobic motif where the presence of a negatively charged glutamic acid in place of a serine mimics a phosphorylated residue. It seems that phosphorylation events are essential for the activity of all PKCs and require the upstream kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) to trans-phosphorylate the activation loop (Balendran et al., 2000).

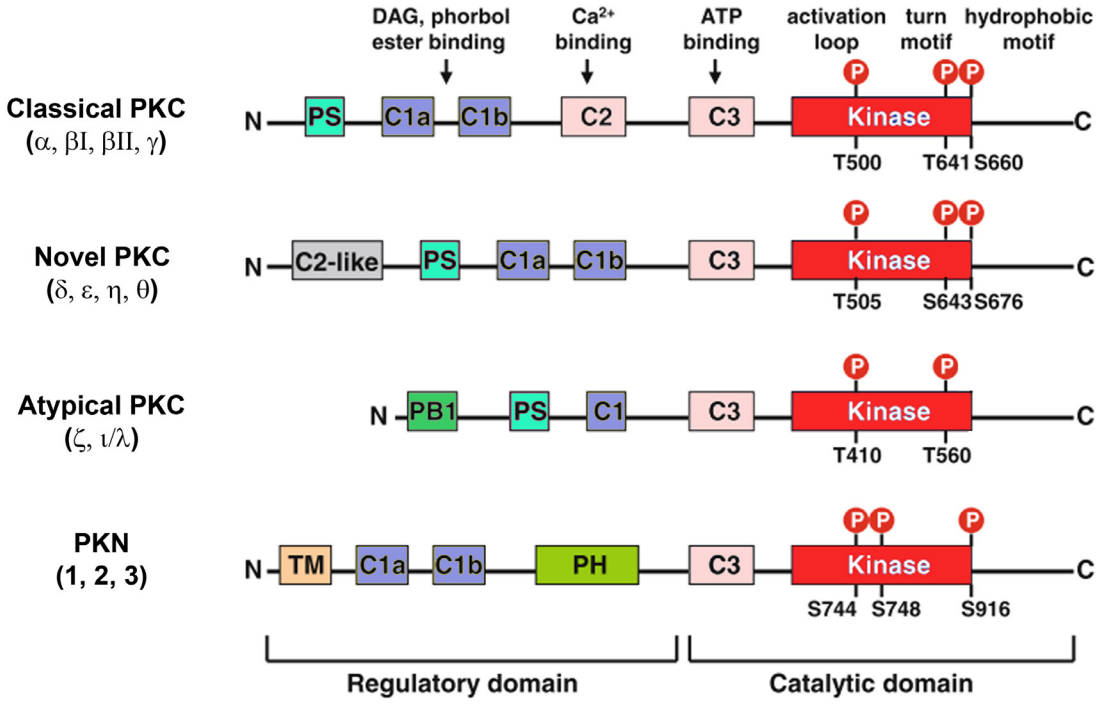

Figure I5. The PKC family. Graphical representation of the four subfamilies of the PKC family with different domains, binding regions within the regulatory domain and phosphorylation sites within the kinase domain.

The current model for the activation of PKCs proposes that this happens through different mechanisms involving phosphorylation and allosteric interactions (Disatnik et al., 1994). In an inactive state the pseudosubstrate region, which is isoform-specific, is buried within the active site of the kinase. The removal of this loop is thought to be preceded by a series of maturation steps through phosphorylation of the kinase domain on three (cPKC and nPKC) or two (aPKC and PKN) residues (Newton, 2003). These steps seem to be necessary for full activation of the PKC family. Two upstream kinases have been reported to play this role. One is PDK1 that phosphorylates the activation loop in the kinase domain, and the other is the mammalian target of rapamycin 2 complex (mTORC2), which regulates phosphorylation of the turn and hydrophobic motifs (when present) (Diaz-Meco & Moscat, 2012).

The activation step takes place in response to binding of lipid second messengers, allosteric effectors, or both, to specific domains at the regulatory region of the PKC. For conventional and novel PKC, increases in plasma membrane DAG levels trigger the membrane recruitment and activation. Thus, allosteric interactions with the membrane seem to activate PKC, but they are thought to be contingent upon a previously phosphorylated kinase domain to occur. Other protein modifications, such as tyrosine phosphorylation or proteolysis, might also be critical factors in mediating PKC activation (Diaz-Meco & Moscat, 2012).

The DAG-insensitive atypical PKCs have been suggested to respond to other lipids such as phosphatidylinositols, phosphatidic acid, arachidonic acid and ceramide (Moscat & Diaz-Meco, 2000). In addition, interaction with specific cellular partners might alter aPKC activity. For instance, the zinc-finger harboured in the C1 domain of aPKCs binds to Par-4 and this interation blocks PKC enzymatic activity (Díaz-Meco et al., 1996). The main site for aPKC modulation by adapters is the PB1 domain, which will be discussed in detail in the next section.

b. Atypical PKC zeta

The atypical protein kinase C (aPKC) subfamily of kinases is composed of two members, PKCζ and PLCλ/i. These proteins are highly related and they share an overall 72% amino acid identity (Nishizuka, 1995). These kinases have been involved in several functions, namely proliferation/survival (Berra et al., 1995) and apoptosis (Díaz-Meco et al., 1996), glucose transport (Liu et al., 2006), inflammation (Diaz-Meco & Moscat, 2012), neuronal differentiation (Wooten, 1999) and in the control of cell polarity (Wu et al., 2007). Additionally, the use of a PKCζ knockout mouse model (PKCζ-/-) has pinpointed a major role in the activation of the transcription factor NFκB (Leitges et al., 2001).

The activation of PKCζ has been reported to involve several alternative or complementary mechanisms (Fig. I6). Standert et al. (2001) reported that insulin provokes increases in PKCζ enzymatic activity through PDK-1-dependent T410 loop phosphorylation, T560 autophosphorylation, and phosphorylation-independent/conformational-dependent relief of pseudosubstrate autoinhibition. Thus, it seems that there is a fraction of PKCζ activation that is not mediated by phosphorylation and represents an unforeseen mechanism for other PKCs. This was suggested after results indicating the phosphomimetic mutant of PKCζ (T410E/T560E) was still able to be further activated by insulin (Standaert et al., 2001). In these lines, Graybill et al (2010) have recently published a mechanism for PKCζ activation mediated by an interaction with Par6. This was achieved by a specific interaction involving the PB1 domains of both proteins, and the C2 domain of Par6, which results in the removal of the pseudosubstrate region from the catalytic site of PKCζ (Graybill et al., 2012). This novel mechanism poses interesting questions on the diverse functions of PB1 domains, which will be covered in detail below. Another phosphorylation-independent mechanism for PKCζ activation is caspase processing. It has been shown that a number of caspases (-3, -6, -7 and -8) process PKCζ to carboxyl-terminal fragments that are catalytically active (Smith et al., 2000). Caspase processing was found to be PKCζ-specific since the highly similar PLCλ lacks the caspase-processing sites. Stimulation with TNFα in HeLa cells promotes PKCζ processing without increasing phosphorylation on T410. Thus, the free kinase domain was found to have higher catalytic activity than the holoenzyme but a shorter half life, which was even increased in phospho-deficient T410A mutants, due to ubiquitin-mediated proteasomal degradation (Smith et al., 2003). Finally, PKCζ is also known to function as a scaffold protein independently of its kinase activity. Diaz-Meco et al (2001) reported that PKCζ was a component of the EGF-initiated pathway for the activation of ERK5 through a direct interaction with MEK5 (see details below in this section). In this system, PKCζ kinase activity was speculated not to be required for this function since the T410A mutant was still able to activate ERK5 (Diaz-Meco & Moscat, 2001). However it remains unclear whether different activation mechanisms other than T410 phosphorylation are operating in this system. Taken together, these data illustrate the diversity of activation mechanisms for PKCζ (Fig. I6) and indicate the need to determine the relative contribution of each of these mechanisms to a given function of the protein.

The PKC family is known to display a relatively low substrate selectively which calls for additional regulation mechanism to confer a high-degree functional specificity. Indeed, the functionality of the aPKC family is highly dependent on accessory and adaptor proteins. p62 provides a useful example to understand the role of these adaptors. This protein was isolated independently by two groups as a novel atypical PKC-interacting partner that binds to the first 126 amino acids upstream of the zinc finger domain (Puls et al., 1997).

Early evidence suggested that p62 provides a scaffold linking the aPKCs to the tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) receptor signalling complexes for the activation of the transcription factor NFκB (Sanz et al., 1999). p62 acted as an adaptor since neither did it affect the catalytic activity of the aPKC nor was it a substrate for the kinase. This scaffold role was found to profoundly affect the function of both PKCζ and PLCλ. The site of p62 to which the aPCKs bind has been narrowed down to a short stretch of acidic amino acids termed AID (for atypical PKC-interacting domain). This domain was later found to be present in a plethora of other signalling proteins (including aPKC) and to form an intrincated protein-protein interaction network. This distinct structural domain was named Phox/Bem 1 (PB1) as it was originally found in the phagocyte oxidase (phox) activator p67phox and the yeast polarity protein Bem1 (Ito et al., 2001).

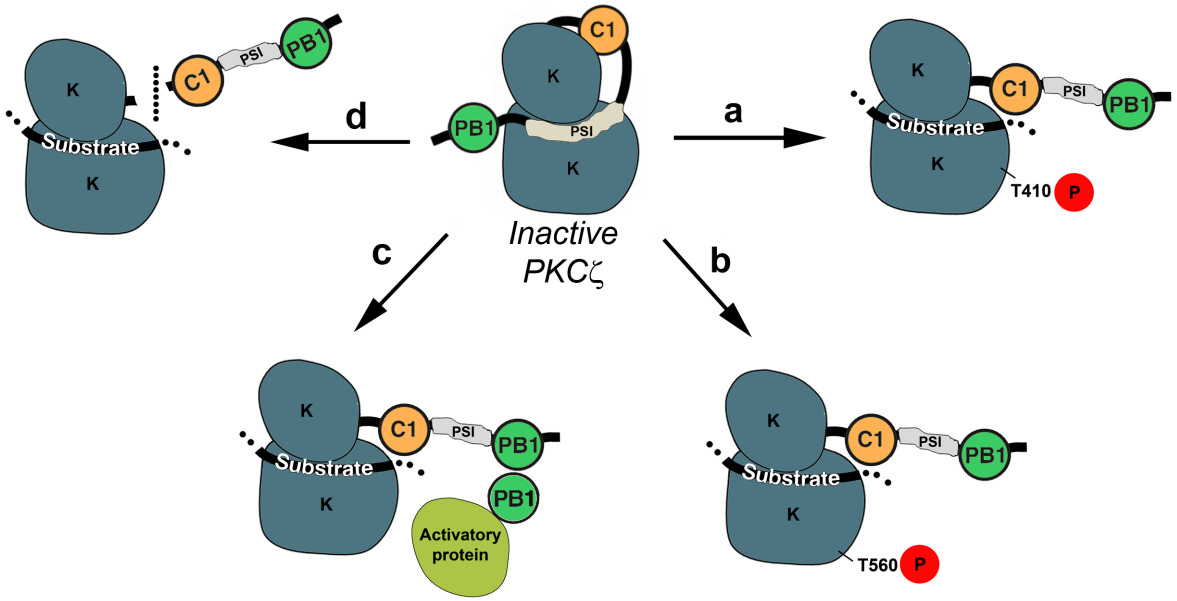

Figure I6. Different activation mechanisms of PKCζ. (A) PDK1-mediated phosphorylation at Thr410. (B) Autophosphorylation at Thr560. (C) Conformation-dependent pseudosubstrate release by PB1-PB1 interactions. (D) Caspase-dependent cleavage of the N-terminus.

The PB1 domain is a protein-protein interaction module conserved in animals, fungi, amoeba and plants. The proteins harbouring this domain, that characteristically adopt a ubiquitin-like β-grasp fold, can be classified into 3 types: PB1-type I proteins are defined by an acidic motif (OPCA); PB1-type II are defined by a predominantly basic motif invariably governed by a lysine on the first β strand; and PB1-type I/II proteins have both types (Sumimoto et al., 2007) (Fig. I7). Homo- and heterodimerisation between PB1 proteins are established through electrostatic interactions between the basic residues of type II

and the acidic residues of type I. The human genome contains at least 13 PB1 domain-harbouring proteins including NADPH oxidase subunits (p67phox and p40phox), atypical PKC signalling modules (PKCζ, PLCλ, Par6, p62), mitogen-activated kinases (MEK5 and its upstream kinase MEKK2), or the scaffold protein Nbr1.

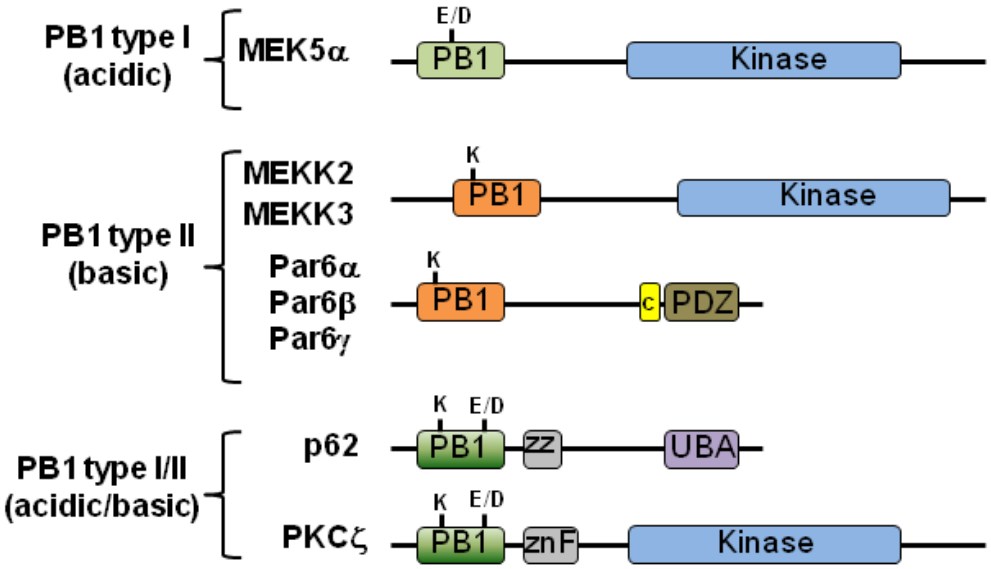

Figure I7. PB1 domains. Graphical representation of the three different types of PB1 domains. PB1-type I proteins harbour an acidic motif (OPCA), PB1-type II harbour a predominantly basic motif invariably governed by a lysine on the first b strand and PB1-type I/II that have both types of motifs.

The PB1 domain in PKCζ is known to participate in several functions through specific interactions with different PB1-harbouring protein partners (Fig. I8). Roughly, these can be divided into three main roles; the control of cell polarity through interactions with Par6 proteins, the control of NFκB function and others through an interaction with p62, and finally, the control of MAPK activation through interactions with MEK5.

Animal cell polarisation involves 6 partition defective (par) gene products, including adaptor proteins Par6 and Par3. From studies carried out in *Caenorhabditis elegans* it was established that the essential complex for polarity was formed between Par-6, Par-3 and PKC-3, the atypical PKC homolog (Macara, 2004). Both aPKC and Par-6 dimerise through their PB1 domains; aPKC acting as a type I (acidic) to interact with Par-6 type II domain (basic). Although, Par-3 does not have a PB1 domain, it is able to interact through other domains with aPKC and Par-6. This complex seems to have a conserved function in polarity from nematodes to mammals. Additionally, it also regulates tight junction formation and determines basal/apical polarity in mammalian epithelial cells (Noda et al., 2003).

An interaction between Par6 and PKCζ also seems to be crucial for Ras activated cystoskeletal remodelling (Coghlan et al., 2000). It is possible that the novel activation mechanism for PKCζ involving PS displacement by Par6 (Graybill et al., 2012) accounts for some of the functions described above.

The aPKC/p62 signalling platform, as mentioned above, is thought to participate mainly in NFκB-activation dependent processes. The pathways leading to the activation of NFκB are diverse. Both IL-1 and TNFα activation of NFκB rely on an interaction between p62 and a specific adaptor, RIP and TRAF6, respectively (Moscat & Diaz-Meco, 2000). This association links the activated receptor with PKCζ which is activated through an unknown mechanism and can then phosphorylate either IKK (NFκB upstream kinase) or the p65 subunit of NFκB, depending on the tissue. Thus, active NFκB will be transported to the nucleus to promote the transcription of important survival genes (Moscat et al., 2009). A clear implication of the p62/aPKC complex has been reported for the regulation of allergic responses and asthma (Soloff et al., 2004). This interaction is required for the differentiation of T cells through a sustained activation of NFκB and for the IL-4-mediated activation of the Jak1/STAT6 pathway (Moscat et al., 2006). Additionally, p62 and PKC participate in the retrograde activation of ERK5 in neurons which will be discussed in the next section. It is noteworthy that p62 is a very polyvalent multidomain protein that is involved in cancer, autophagy and apoptosis (Moscat & Diaz-Meco, 2009). However, the requirement for aPKC/p62 interaction in these processes is not clearly established. This is the case for the characteristic cellular speckles in autophagy formed of aggregated proteins in the cytoplasm. There is preliminary evidence for an increase of p62 aggregates in the absence of aPKC/p62 interaction (Puls et al., 1997), but further evidence is required to determine aPKC function in autophagy. Also, the contribution of p62 and PKCζ to cancer progression and apoptosis is diverse and often they play opposing roles which suggest independent functions in these complex processes (Moscat et al., 2009).

Another PB1-dominated process is the activation of the MAPK ERK5, which will be covered in detail in the next section. MEKK2 and MEKK3 are the upstream kinases of MEK5, which is in turn responsible for the phosphorylation of ERK5. Both MEKK2/3 and MEK5 harbour a PB1 domain through which they establish interactions that lead to the activation of MEK5. The PB1 domain in MEK5 is also capable of interacting with ERK5, which does not have a PB1 domain, resulting in the phosphorylation of the MAPK (Nakamura & Johnson, 2003; Nakamura et al., 2006). The involvement of PKCζ in this process became clear when it was reported that it directly interacts with MEK5 and is required for the activation of ERK5 by EGF (Diaz-Meco & Moscat, 2001). This interaction is also established through PB1 domains and results in an enhancement of MEK5 kinase activity that

then becomes autophosphorylated. The activation of MEK5 in this system is likely due to allosteric effects of PKCζ binding, since the kinase activity of the latter is not required. More recently, we reported that this same signalling module was utilised by Gq-coupled GPCR to activate the ERK5 pathway (see section 5e).

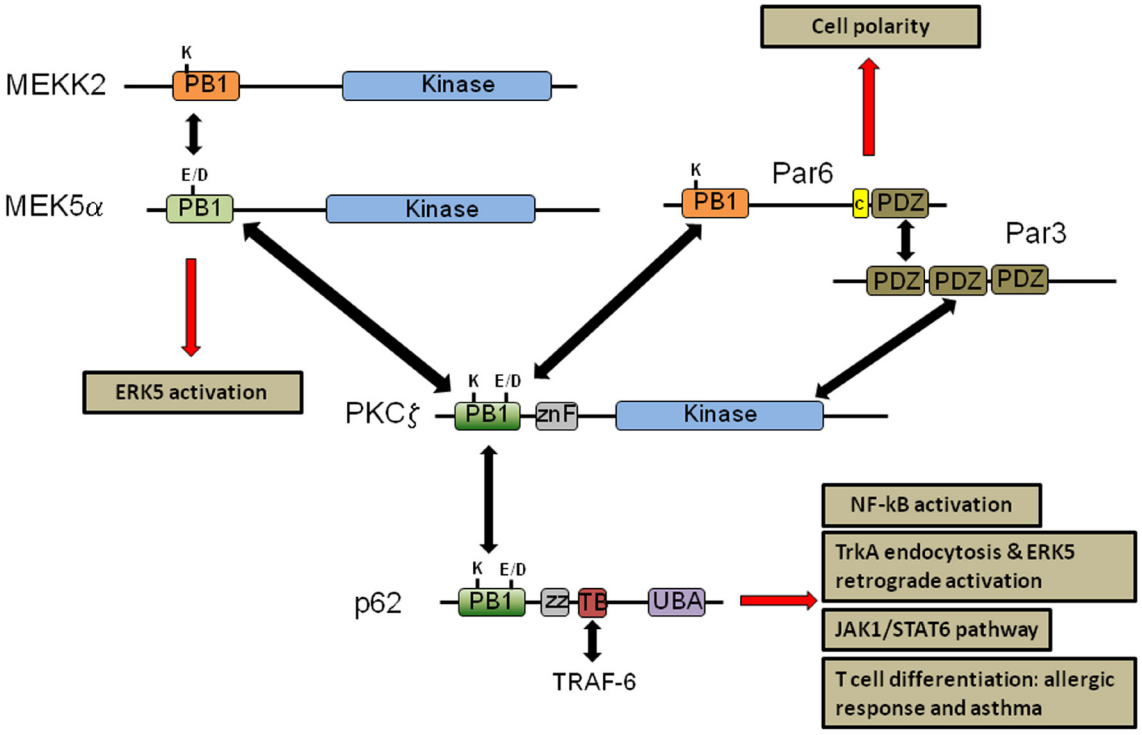

Figure I8. PKCζ-PB1 domain functions. The PB1 domain of PKCζ participates in several functions through different protein-protein interactions: Control of cell polarity through interactions with Par6 proteins, the control of ERK5 activation through interactions with MEK5 and the control of NFκB and other functions through an interaction with p62.

4. MITOGEN-ACTIVATED KINASES

a. General features

Members of the family of mitogen-activated protein kinases (MAPKs) are proline-targeted serine/threonine kinases that play an essential role in signalling through the modulation of gene transcription in the nucleus in response to changes in the cellular environment. MAPKs control key cellular functions, including proliferation, differentiation, migration and apoptosis, and participate in a number of disease states including chronic inflammation and cancer (Turjanski et al., 2007). In humans, there are at least 11 mem-

bers of the MAPK superfamily, which can be divided into six groups based on sequence similarity: the extracellular signal-regulated protein kinases (ERK1 and ERK2); c-Jun N-terminal kinases (JNK1, JNK2, JNK3); p38s (p38a, p38b,p38g, p38d); ERK5 (ERK5); ERK3s (ERK3, p97 MAPK, ERK4) and ERK7s (ERK7, ERK8) (Pearson et al., 2001). Each subfamily of MAPKs can be stimulated by a separate protein kinase cascade that involves the sequential activation of a specific MAPK kinase kinase (MAPKKK) and a MAPK kinase (MAPKK), which in turn phosphorylates and activates downstream MAPKs (Fig. I9). For complete activation MAPK ought to be phosphorylated in two highly conserved residues, serine and threonine, located in the activation loop. It seems that specificity between different subfamilies is achieved through an intercalated residue between the two phosphorylation sites; glutamic acid in ERK1/2 and ERK5 (TEY motif), glycine in p38 (TGY motif) and proline in the case of JNK (TPY motif) (Turjanski et al., 2007). These signalling modules have been conserved throughout evolution, from plants, fungi, nematodes and insects to mammals (Widmann et al., 1999). MAPK research has grown exponentially over the last two decades (more than 3000 articles published in 2012) to become a paradigmatic example of the cell signalling field.

b. Activation of classical MAPKs by Gαq

The activation of Gαq/11 family of G proteins triggers different signals that have been implicated in the control of the ERK1/2, p38 and JNK cascades.

Gαq-mediated PKC activation leads to ERK1/2 activation through both Ras-dependent and Ras-independent mechanisms. PLCα can activate Raf-1 by direct phosphorylation (Kolch et al., 1993), and stimulation of ERK by the Gαq/11-coupled α1 adrenergic and M1 muscarinic receptors in fibroblasts can occur through a Raf-dependent, but Ras-independent, mechanism that is blocked by PKC downregulation and mimicked by overexpression of PLCβ2 (Faure et al., 1994). In many cases, however, Gαq/11 activation causes ERK1/2 activation independently of PKC. Two of the most common PKC-alternative mechanisms are receptor transactivation and calcium-induced responses. In fibroblasts, shedding of the extracellular ligand HB-EGF is promoted upon stimulation of Gαq/11-coupled receptors (endothelin-1 and α-thrombin receptors). Despite the fact that the MMP ADAM-9 mediates PKC-dependent HB-EGF cleavage in response to phorbol esters, the Gαq/11 effect, like that mediated by Gβγ subunits, apparently involves neither PKC nor ADAM-9 (Prenzel et al., in press). In cultured vascular smooth muscle cells, transactivation of PDGF-B receptors apparently accounts for ERK activation in response to stimulation of the Gαq/11-coupled angiotensin AT1a receptor (Linseman et al., 1995). Additionally, calcium-dependent mechanisms may play a role in Gαq/11-mediated ERK activation,

particularly in cells of neuronal origin. Gαq/11-dependent activation of the calcium- and cell adhesion-dependent focal adhesion kinase (FAK)-family member, Pyk2, leads to Ras-dependent ERK1/2 activation. In this context, intracellular calcium, released as a result of PLCβ-mediated inositol phosphate production, triggers Pyk2 autophosphorylation, recruitment of the non-receptor tyrosine kinase c-Src, tyrosine phosphorylation of Shc, and Ras-dependent ERK1/2 activation (Dikic et al., 1996).

In vivo studies have also shown the involvement of Gαq in the activation of JNK (Minamino et al., 2002). As it was the case for ERK1/2, diverse mechanisms operate in the activation of JNK downstream of Gq-coupled receptors. Both DAG- and calcium-mediated pathways can activate JNK, in a process that requires Src and Pyk2 activation, respectively, of Rac-1 or R-Ras. These monomeric GTPases activate specific MAP4K, such as MLK3, that lead to the initiation of the JNK cascade (Goldsmith & Dhanasekaran, 2007). Ask1 is the main MAPKKK in the activation of both JNK and p38 pathways by GPCRs. The activation mechanisms of p38 MAPK by Gq-coupled GPCRs are similar to those described for JNK. The release of DAG and calcium activates PKC that, in turn, activates Src. Subsequently, Src activates GEFs that will then promote GTP binding in monomeric G proteins. Two specific GEFs for Rac have been reported to be activated by Src: Tiam and Ras-GRF (Buchsbaum et al., 2002).

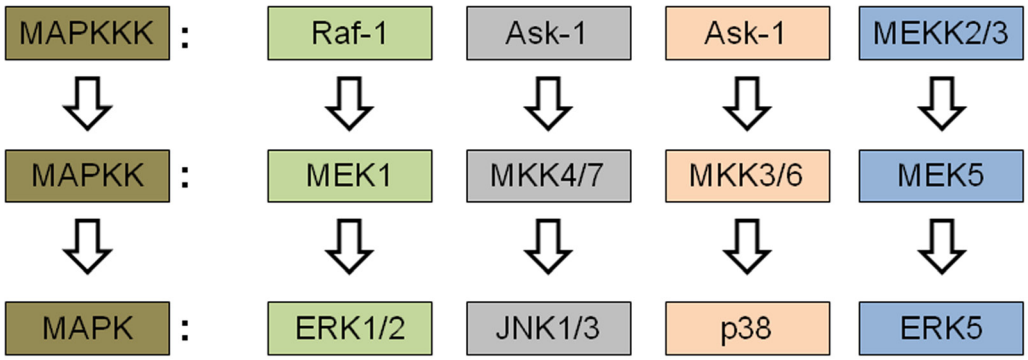

Figure I9. MAPK cascades. Mitogen-activated protein kinase (MAPK) cascades are phospho-relay systems in which a MAPK kinase kinase (MAPKKK) is phosphorylated and, in turn, phosphorylates a MAPK kinase (MAPKK), which leads to the phosphorylation of a MAPK. Main MAPK cascades (ERK1/2, p38, JNK, ERK5) are depicted here.

c. The extracellular signal regulated kinase 5 (ERK5) MAPK

The ERK5 MAPK is classically activated by different stimuli like mitogens (EGF, granulocyte-colony-stimulating factor), cytokines (leukemia inhibitory factor, cardiotrophin-1), and stress (Hayashi & Lee, 2004; Obara & Nakahata, 2010; Wang & Tournier, 2006). Also, Gq-coupled GPCRs are known to activate ERK5 thereby regulating c-jun expression through the activation of members of the MEF2 class of transcription factors

(Marinissen et al., 1999). However, because of the relative paucity of investigations on this pathway, there are fewer known upstream activators described for ERK5 compared to the other families (ERK1/2, p38 and JNK) of MAPK (Rose et al., 2010).

Although ERK5 is phophorylated in TEY, the same consensus motif as ERK1/2, it is structurally very different to the latter. ERK5 contains a unique loop-12 structure and an unusually large C terminal domain which confers this protein a large molecular weight (115KDa)(Hayashi & Lee, 2004). Hence, ERK5 is also known as big MAPK 1 (BMK1). The MAPK signalling module of ERK5 is composed of three sequentially activated kinases: upstream kinases (MAPKKK) are MEKK2 and MEKK3 that activate MEK5 (MAPKK), which is the direct activator of ERK5. Indeed, a distinctive feature of this pathway is the high specificity conferred by MEK5 since ERK5 is its only known substrate. Thus, any signal converging into the activation of MEK5 will only result in the activation of ERK5. On the contrary, MEKK2 and MEKK3 are not specific for the ERK5 pathway for they are known activators of JNK and p38 (Deacon & Blank, 1999). Phosphorylated ERK5 has been reported to act on several nuclear substrates including the myocyte enhancer 2 (MEF2) family of transcription factors, Sap1a (Ets-domain transcription factor), connexin 43 (a gap junction protein) or the pro-apoptotic protein Bad (Roberts et al., 2009) (Fig. I10).

The activation mechanism of ERK5 illustrates a number of distinctive features of this kinase in comparison to the rest of MAPKs (Fig. I10). According to the classical view, inactive ERK5 resides in the cytoplasm and it translocates to the nucleus upon phosphorylation (Esparís-Ogando et al., 2002). However, this does not seem to be always the case since nuclear localisation of ERK5 has been reported in unstimulated cells and also active MEKK2 has been shown to translocate to the nucleus, suggesting that ERK5 activation might also occur within this cellular compartment (Raviv et al., 2004). Additionally, the large C terminal region of ERK5 confers unique functional proprieties that are not found in any other MAPK. It is required for maximum activation of most ERK5 substrates and, most strikingly, this domain alone has the ability to increase transcriptional activity (Kasler et al., 2000). It is proposed that interactions of ERK5 C-Terminal domain with transcription factors increases their transcriptional activity up to 50-fold. The C terminal domain is known to fold over the kinase domain in an inactive conformation. Upon phosphorylation of the TEY motif, the kinase domain is activated and it transphosphorylates the C terminus that is then released. Although the precise dynamics of the activation are not well understood it seems that ERK5 would be able to display both functions (kinase and transcriptional) simultaneously in the nucleus.

ERK5 characteristic pro-survival/anti-apoptotic function is involved in a number of important patho-physiological processes such as carcinogenesis, neuronal survival, en-

dothelial integrity and cardiovascular function (specific involvement of ERK5 in the heart will be covered in 6c). Cancer is characterised by deregulated signalling and aberrant response to mitogenic stimuli. As it is the case for all other MAPKs, ERK5 has been associated with various malignant properties of human cancers including augmented metastatic potential of prostate cancer, sustained growth of ErbB2-positive breast carcinomas and chemoresistance of breast cancer cells (Hayashi & Lee, 2004). Also, the overexpression of MEK5 correlates with bone metastasis possibly due to an increased de novo synthesis of matrix metalloproteinase 9 that degrades extracellular matrix thus facilitating the invasion and spreading of cancer cells (Mehta et al., 2003).

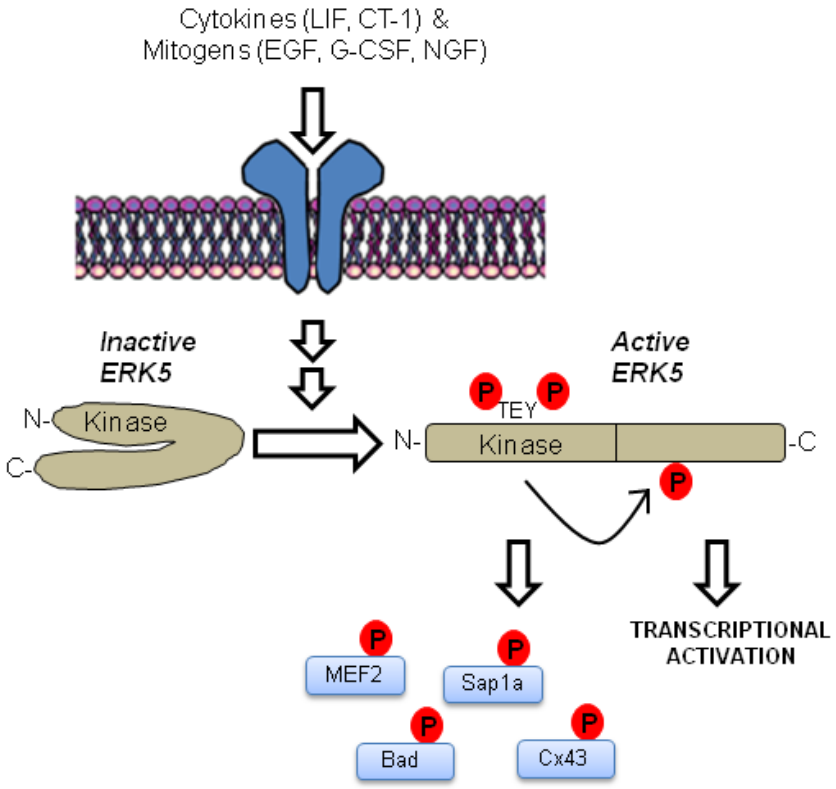

Figure I10. Classical ERK5 activation pathways. Ligands binding to tyrosine kinase receptors lead to activation of ERK5 by promoting a dual phosphorylation on the kinase domain (TEY motif). Activated ERK5 phosphorylates a number of substrates. Autophosphorylation of the long C-terminus promotes the transcriptional activation function.

Growth factors synthesised and released by target tissues promote survival of innervating neurons. This retrograde survival response is initiated when growth factors bind receptors at nerve terminals which are internalised and transported via the endocytic machinery through the axon to the soma, where they activate different cascades. Neurotrophins are the growth factors involved in retrograde signalling and they act through receptors termed Trks on the nerve terminals. It has been shown that ERK5, but not ERK1/2, is essential for this retrograde signalling and for neuronal survival (Watson et al., 2001). This process was found to be dependent on the PB1 domain-dominated interaction bet-

ween p62 and aPKC (Samuels et al., 2001). Indeed, p62 was shown to establish an interaction with TrkA and TRAF6 that promoted receptor internalisation and subsequent ERK5 activation (Geetha & Wooten, 2003).

The analysis of tissue-specific ERK5 knockout mice has suggested that this kinase is essential for the development and normal function of the endothelium. ERK5-null mice die at embryonic day 10 due to cardiovascular defects due to a failed maturation of cardiac vasculature. Indeed, cardiomyocyte-specific ERK5-KO mice develop normally whereas endothelium-specific mice recapitulate the global knockout defects (Hayashi et al., 2004). Also, neuron-specific KO develop normally which, together with the rest of the knockout data, suggests that compensatory mechanisms operate in all tissues were ERK5 is acting except from the endothelium where it seems to be indispensable. Endothelial ERK5 is activated by stress and induces the phosphorylation and inactivation of the pro-apoptotic protein Bad which prevents its nuclear translocation and caspase 3 activation (Pi et al., 2004). Interestingly, PKCζ is a known pro-apoptotic factor activated by shear flow stress in the endothelium which results in the down-regulation of ERK5-initiated eNOS pathway. PKCζ was reported to directly interact with and phosphorylate ERK5 on serine 486 thereby inhibiting its transcriptional activity (Nigro et al., 2010). However, no evidence for the inhibition of ERK5 kinase activity was shown. Besides, the presence of p62 has been determined crucial for this process (Kim et al., 2012). This negative regulation is a puzzling finding, possibly endothelium-specific, since PKCζ has been extensively shown by us (see section 5e, 6c and results) and others to be necessary for ERK5 activation by a number of stimuli (Diaz-Meco & Moscat, 2001; García-Hoz et al., 2010, 2012). However, it is clear that ERK5 function is highly interconnected to that of PKCζ and other PB1 domain proteins. Since PB1 domains are known to serve as signalling organising platforms it is likely that other proteins are present in the active complexes with ERK5. Thus, the description of the differential cellular determinants that account for the surprising opposing roles described above clearly deserves further investigation.

ERK5 is one of the numerous proteins activated downstream of Gαq. All these pathways emanating from the activated G protein rely on specific interactions between Galphaq and a myriad of cellular effectors. This subject will be covered in detail in the following section.

5. THE Gαq INTERACTOME

A number of cellular proteins have been reported to interact with Gαq. The effect on Gαq signalling achieved by these interactions can be activatory, if as a result of the interaction Gαq signal is propagated or inhibitory, if the opposite effect is achieved and Gαq is

deactivated (Fig. I11). The effectors belong to the first type and are proteins whose activity depends on direct association with GTP-bound Galphaq *in vivo* in response to receptor activation. Other activatory interactors are scaffold molecules that organise and facilitate Gαq signalling. The second type of interactors are named regulators and they limit Gαq signalling by enhancing the GAP activity of the G protein or by sequestering it and preventing effectors to interact. Although most Gαq functions are tied to the activation of one or more effectors, to date there still are Gαq-dependent processes for which no effector has yet been identified. Such is the case of the activation of the glycogen synthase kinase-3 (GSK-3) promoted by Gαq independently of PLCβ (Fan et al., 2003). A brief description of most relevant Gαq functional interactions is included below and summarised in Figure I11.1.

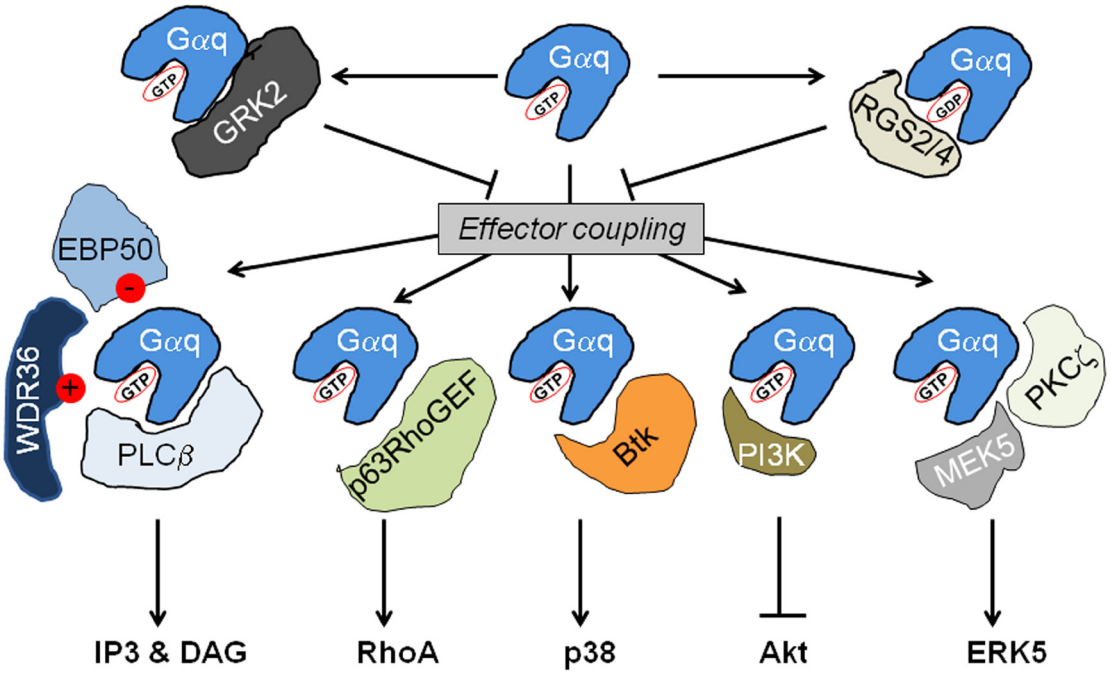

Figure I11. Gαq effectors and regulators. Gαq versatile binding potential allows for a diverse functional activity. Gαq can bind to PLCβ (and adaptor proteins WDR36 and EBP50), p63RhoGEF, Btk, PI3K, PKCζ and MEK5. Gprotein-effector complexes are responsible for the activation of different pathways. The formation of these complexes is negatively regulated by GRK2 and RGS proteins.

a. Gαq initiates lipid and calcium-dependent signalling through PLCβ

Phospholipase Cs are Ca^{2+}-dependent enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) to yield inositol 1,4,5 trisphosphate ($Ins\ (1,4,5)P_3$) and diacylglycerol (DAG), which are important secondary cellular messen-

gers (Rhee, 2001). These enzymes can be found at all stages in evolution from the simplest bacterial PLC consisting only of the catalytic domain which requires Ca^{2+} for activity to the complex multidomain organization of mammalian PLC (Drin & Scarlata, 2007). The PLCβ family has four isoforms (1–4) all of which can be stimulated directly (although with different potency) by both Gαq-GTP and Gβγ to hydrolyze $PI(4,5)P_2$ and trigger inositol lipid and calcium-mobilising signalling (Rhee, 2001). The involvement of PLC activation in mobilization of intracellular Ca^{2+} and activation of protein kinase C in response to many GPCR ligands was elucidated in the 1970s and early 1980s (Harden et al., 2011). Later it was shown that PLCβ could be activated by GTP in cell-free preparations, however, it was not until 1991 that a recently discovered class of G proteins, the Gαq/11 family was reported to directly and specifically activate PLCβ isozymes ((Harden et al., 2011)and references therein).

Signal transfer between Gαq and PLCβ is tightly regulated by these two proteins in a coordinated process of activation/deactivation. The particular structural properties of PLCβ confers it the ability to both be activated like an effector and to act on the G protein promoting its GAP activity and deactivation (Waldo et al., 2010b) (see section 2c). Thus, the capacity of Gα-subunits to hydrolyze GTP and therefore self-regulate their activation state is aided by PLCβ. On the other hand, controversial reports have put forward the possibility that Gαq protein needs to be phosphorylated on its C-terminus (tyrosine 356) to reach full activation of PLCβ in response to carbachol (Liu et al., 2002; Umemori et al., 1997).

b. Gαq-mediated Rho activation by RhoGEFs

The Rho GTPases are a family of peripheral membrane proteins that regulate important cellular processes such as cell shape, cell migration, cell cycle progression, and gene transcription (Etienne-Manneville & Hall, 2002). RhoA, Rac1 and Cdc42 are the best characterized members of this family, known to control the dynamics of the actin cytoskeleton and to stimulate gene transcription through several transcription factors, such as the serum response factor (SRF) or nuclear factor κB(NFκB)(Ridley, 2006). The activation of these small GTPases can be enhanced by their interaction with Rho-guanine nucleotide exchange factors (RhoGEFs) that catalyze the exchange from protein-bound GDP to GTP. RhoGEFs increase the speed of the rate-limiting step, GDP dissociation, which is followed by GTP occupation of the nucleotide-binding pocket, a more favourable process due to the higher cytosolic concentration of GTP (10:1 ratio)(Bos et al., 2007). RhoGEFs can serve as direct downstream effectors of heterotrimeric G proteins thus linking GPCR activation to Rho GTPases. The Gαq/11 family has been shown to activate RhoA, albeit with a lower potency than proteins of the Gα12/13 family (Vogt et al., 2003).

The first candidate suggested to participate in this activation through a direct interaction with Gαq was the leukemia associated Rho guanine nucleotide exchange factor (LARG) (Booden et al., 2002). Since LARG possesses an RGS box region, a known Gαq interacting domain present in the regulators of G protein signalling family (section 5h), this region was initially proposed to participate in the association with Gαq, although the subject is currently under discussion (Booden et al., 2002; Chikumi et al., 2002; Fukuhara et al., 2000). Also, other RhoGEFs without an RGS domain such as Trio, Duet and p63RhoGEF are known to interact with Gαq and promote RhoA activation (Lutz et al., 2005, 2007; Rojas et al., 2007; Williams et al., 2007). Lbc, another RhoGEF family member was also shown to bind Gαq but no cellular effects of the interaction have been described (Sagi et al., 2001).

p63RhoGEF is the best characterised non canonical and selective effector of Gαq. A crystal structure of the complex revealed the mechanism underlying the activation of RhoA by this heterotrimeric G protein subunit (Lutz et al., 2007). Interestingly, p63RhoGEFand PLCβ have been described to inhibit each other´s downstream responses (serum response element activation and IP3 production, respectively) thus providing an interesting model for the competition between alternative effectors following G protein activation (Lutz et al., 2005). Although the authors did not directly show whether this mutual inhibitory effect was due to competition for the Gαq binding site, it seems likely since they both bind to a similar surface of the G protein (Waldo et al., 2010a).

A regulatory mechanism for Gαq-induced Rho activation was recently revealed. It involves a direct interaction between the mitogen-activated kinase MLK3 and p63rhoGEF that prevents Gαq from associating with the latter thereby abrogating Rho activation. This process happens in a MLK3 kinase activity-independent manner and seems to regulate important Rho-mediated functions such as cell migration (Swenson-Fields et al., 2008).

c. Gαq activates the p38 pathway through Btk

Bruton tyrosine kinase is a protein that appears mutated in the immunodeficiency disease X-linked α-gammaglobulinaemia and was one of the first non-phospholipase effectors of Gαq to be suggested. *In vitro* experiments showed that Gαq, but not Gi1, Go or Gz, interacts with the kinase and promotes its autophosphorylation (Bence et al., 1997). The authors also showed that Btk was necessary for the activation *in vivo* of p38 MAPK by Gq-coupled receptors. Subsequent work mapped out the interface on Btk for association with Gαq and proposed an activation model that entailed the disruption of an intramolecular inhibitory interaction (Ma & Huang, 1998), much in the lines of the later-to-be described activation mechanism for other effectors (section 2b). However novel at the time,

these findings have not been substantiated with additional work on the cellular repercussions of the interaction and extensive reviews devoted to Btk even fail to mention the link with Gαq (Lindvall et al., 2005; Mohamed et al., 2009).

d. Gαq modulates PI3K and the Akt pathway

Phosphatidil-inositol 3 kinase (PI3K) has also been reported to be regulated by Gαq. First evidences showed that the activation of PI3K and Akt by insulin growth factor 1 (IGF-1) and platelet derived growth factor (PDGF) were inhibited by the Gq-coupled receptor α1-adrenergic thus promoting apoptotic responses (Ballou et al., 2000). Later it was shown that activated Gαq inhibited p110 PI3K and Akt, independently of PLCβ (Ballou et al., 2003). This effect was suggested to be due to a direct and inhibitory effect of GTP-bound Gαq on p110α/p85α PI3K that competes with Ras, a PI3K activator (Ballou et al., 2006). This was the first account of a Gαq interaction that played a negative role on the effector. However, it is noteworthy that it has been speculated that the effect of Gαq on the Akt pathway can vary depending on the cell type. Indeed, several reports have shown that Gq-coupled receptors such as bradykinin, thrombin or carbachol induce Akt phosphorylation in a variety of cell types ((Xie et al., 2000) and references therein). Also, Gαq is proposed to be upstream of PI3K in the pathway that leads to the translocation of the glucose transported GLUT4 (Imamura & Vollenweider, 1999). A link between PI3K and Gαq has not been established in this system.

Since Gαq can either be a proliferative factor or induce apoptosis (see section 6), it is tempting to suggest that Gαq-mediated regulation of the PI3K/Akt pro-survival cascade and of other additional pathways will drastically vary in different cellular contexts.

e. Gαq activates ERK5 through PKCζ and MEK5

Until very recently, the GPCR-initiated pathway leading to ERK5 was unknown as the classical routes for the activation of MAPK by GPCR (involving Ras, Rho, Rac, and/or Cdc42) did not seem to be involved (Fukuhara et al., 2000). In 2010 our group described the molecular mechanism for the activation of ERK5 by the Gq-coupled M1 muscarinic receptor in epithelial cells (García-Hoz et al., 2010). The activation did not require PLCβ activity as demonstrated by the unaffected phosphorylation of ERK5 upon treatment with the specific PLCβ inhibitor U73122. In fact, ERK5 was activated by a constitutively active mutant of Gαq and this was also true for an active Gαq mutant unable to interact with PLCβ (GαqQ209L-R251A/T252A, characterised in (Venkatakrishnan & Exton, 1996)). We found that the atypical protein kinase C zeta (PKCζ), previously reported to participate in the activation of ERK5 by the EGF receptor, was required for the new pathway. Indeed,

Gq-coupled receptors were not able to activate ERK5 in PKCζ-deficient cells or upon pharmacological inhibition of the kinase. PKCζ was reported to interact directly and in a stimulus dependent manner with Gαq, thus favoured by the active state of the G protein. This interaction is highly specific since neither other G alpha family members nor PLCλ, the other member of the atypical PKC family could interact with PKCζ and Gαq, respectively. MEK5, the activatory upstream kinase of ERK5, was also present in the multimolecular complexes of activated Gαq and, surprisingly, it associated directly and in an activation-dependent manner with this G protein subunit. Thus, Gαq was found to bind to two novel effectors in the activation of ERK5. Analysis of the time courses for these associations and of the different combinatorial possibilities of protein complexes revealed a previously unreported adaptor role for Gαq. The G protein sequentially binds to both effectors forming a transient ternary complex and allowing for PKCζ and MEK5 to interact and activate ERK5 (Fig. I12). The necessary transient character of the complex was revealed by a mutant form of MEK5 (MEK5δAID) that cannot interact with PKCζ but preserves its Gαq-binding ability. The Gαq/PKCζ complex, usually disrupted by MEK5wt, was however stabilised by MEK5dAID and this resulted in an abrogated ERK5 activation (García-Hoz et al., 2010). Thus, our recent work put forward PKCζ and MEK5 as two novel Gαq effectors that participate in ERK5 activation by GPCR.

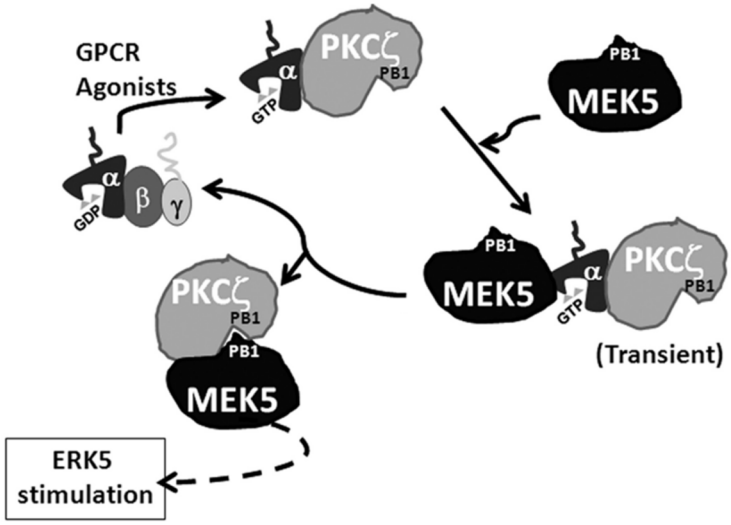

Figure I12. Working model for ERK5 activation by Gq-coupled GPCR. Activated Gαq forms a complex with PKCζ, the first effector of the pathway. MEK5 is attracted into the complex through a direct interaction with Gαq and forms a transient ternary complex. This is stabilised by a MEK5 mutant with no PB1 domain (MEK5dPB1). MEK5 rapidly binds PKCζ and displaces Gαq from the complex. This is inhibited by PKCζ-PB1 domain. PKCζ allows for MEK to bind and phosphorylate ERK5 (García-Hoz et al., 2010).

f. Accessory proteins in effector coupling

G protein-effector complexes are not isolated entities as they are usually depicted in classic text-book biochemistry but are instead supported by numerous other proteins playing accessory roles. These complexes might be confined to very specific locations and the aid of scaffolding proteins can be essential for effector coupling and effective signalling (also see next section).

An interesting example of this was recently reported by Cartier et al (2011). WDR36 was identified as an interacting partner of the tromboxane A2 receptor β type (TPβ) and this association was positively modulated by receptor stimulation. Additionally, it was found that WDR36 could also interact with Gαq and PLCβ, thus acting as a scaffold and tethering receptor, G protein and effector within the same active complex (Cartier et al., 2011). Besides, the scaffold competes for the binding site of GRK2 in Gαq and prevents deactivation by the kinase. The presence of WDR36 was indeed shown essential for Gαq signalling and disease-associated variants of the protein proved unable to fully activate the G protein. Another example is provided by CD9 and CD81 proteins, members of the tetraspanin family that act as protein organizers for extensive signalling complexes on the membrane. They were found to interact with the GPCR GPR56 and with Gαq (Little et al., 2004) thereby facilitating receptor-G protein coupling. This mechanism was not dependent on lipid rafts or other microdomains and illustrates the variability of signalling microenvironments for Gαq.

Scaffold proteins can also play negative regulatory roles. Ezrin-radixin-moesin-binding protein 50 (EBP50) is a multifunctional adaptor that was found to interact with activated Gαq and inhibit PLCβ signalling (Rochdi et al., 2002). EBP50 also blocks the Gαq-induced internalisation of the TPb receptor (Rochdi & Parent, 2003). The study of this inhibitory relationship could provide some clues on PLCβ-independent signalling by Gαq since alternative pathways could be emanating from these complexes.

g. Cellular microenvironments in Gαq signalling

The study of localisation of Gαq in diverse signalling microenvironments could help understanding the complex effector coupling potential of the G protein. Gαq is known to interact with components of the cytoskeleton such as tubulin or filamentous actin (F-actin) as well as with important organisers of membrane microdomains such as caveolin-1 and flotillin.

G proteins can regulate the microtubule cytoskeleton, thus affecting many aspects of cell morphology, by direct binding rather than or in addition to impacting the cytoskeleton through the modulation of downstream signalling pathways (Hewavitharana & Wedegaertner, 2011). Several heterotrimeric G proteins are known to interact with high affinity with tubulin (Wang et al., 1990). The effects of these interaction are antagonistic; whereas G proteins (Gαi1, Gαs and Gαq) destabilize the microtubules by stimulating tubulin´s GTPase activity, Gβγ subunits promote microtubule stability (Dave et al., 2009). Also, the localization of the protein complexes differs between the G protein subtypes. Whereas Gαs, the only G protein known to internalize, seems to interact with tubulin in the cytosol (Sarma et al., 2003) directed by lipid rafts-derived vesicles (Yu & Rasenick, 2002), the stimulation of Gq-coupled receptors appears to recruit tubulin to the membrane (Ciruela & McIlhin-

ney, 2001). This interaction occurs in a similar time course to PLCβ activation (Popova & Rasenick, 2003) although no causal relationship has yet been established between these two phenomena. Additionally, G proteins also interact with the actin cytoskeleton. Gαq/11 was reported to colocalise with F-actin and disruption with the drug cytochalasin inhibited Gαq/11-mediated generation of IP_3 in cells (Ibarrondo et al., 1995).

Thus, there is a cooperative relationship between cytoskeletal components, GPCRs and G proteins to confine signalling molecules in specific microdomains. Particularly, tubulin is an important component of lipid rafts, membrane microdomains comprised of a dynamic assembly of cholesterol and sphingolipids (Simons & Toomre, 2000). Both GPCRs and G proteins are often found in these detergent-resistant domains at higher concentration than elsewhere in the membrane. Indeed, Gαq is often used as an experimental marker of lipid rafts enriched in caveolae (Qiu et al., 2011) whereas other G proteins like Gαi or Gαs target lipid rafts with abundant glycosylphosphatidylinositol (GPI)-anchored proteins. Caveolin 1, a major protein component of lipid rafts, is known to interact with Gαq and modulate dependent Ca^{2++} signalling (Sengupta et al., 2008). Also, caveolin 1 is known to modulate Gαq-signalling through interactions with receptors such as the 5-HT2A serotonin receptor (Bhatnagar et al., 2004). Additionally, G proteins can traffic in and out of lipid rafts upon receptor stimulation thus revealing the important functional implications of these membrane domains. Chronic stimulation of the thyrotropin-releasing hormone receptor induces Gαq to exit detergent-resistant membranes (Pesanová et al., 1999).

Flotillins are other lipid rafts resident proteins that are known to interact with Gαq. The authors found that this protein participated in the activation of p38, but not ERK1/2, by the Gq-coupled receptor PY2 (Sugawara et al., 2007).

h. RGS proteins promote Gαq signal termination

Duration of G protein signalling is limited by the rate of GTP hydrolysis. After returning to the GDP-bound state, Gαq reforms the complex with Gβγ and becomes available for another activation round. The rate of hydrolysis is naturally low and can be accelerated by interaction between GTPase-activating proteins (GAPs) and the G protein. Although some effectors, such as PLCβ, are known to accelerate GTP hydrolysis, the main group of GAPs for G proteins belong to the Regulators of G protein signalling (RGS) family (Watson et al., 1996).

The rate of GTP hydrolysis by Gαq can be accelerated by many different RGS proteins (Hubbard & Hepler, 2006), but two of the best characterized are RGS2 and RGS4, both of which are members of the RGS B/R4 subfamily (Bansal et al., 2007). In cells, RGS4 inhibits both Gαi- and Gαq-mediated signalling, whereas RGS2 is selective for Gαq (Xu et al., 1999).

As previously discussed, PLCβ has evolved to display both effector and GAP functions within the same protein so it is likely that the regulation of PLCβ signalling by RGS proteins will differ from that exerted on other effectors. Indeed, while PLCβ binding clearly overlaps with RGS binding region and regulation is likely due to competition, the binding of p63rhoGEF and GRK2 to Gαq seems compatible with that of RGS. Thus, it has been proposed that RGS proteins form ternary complexes with G proteins and effectors with little or no interaction between RGS and effectors. A recent report shows a mutual allosteric regulation between the effector and RGS binding sites (Shankaranarayanan et al., 2008). In these ternary complexes both RGS2 and RGS4 negatively regulate the binding of GRK2 and p63rhoGEF to Gαq. Besides, RGS protein activity was also affected by effectors although marked differences were observed between the different subtypes. Most importantly, the Gαq-mediated enhanced activity of the effector p63rhoGEF was allosterically reduced by RGS proteins (Shankaranarayanan et al., 2008). The authors speculated on the possible physical conduit of this allosterism and pointed at N-terminus of the switch II of Gαq, a domain sensitive to nucleotide binding and located in between the effector and GAP binding interfaces. Thus, a dual negative regulation is exerted by RGS proteins on Gαq: GTPase activity enhancement and negative allosterism on effector binding. This last mechanism explains why RGS2/4 can inhibit PLCβ-induced activity promoted by a nonhydrolyzable GTP analog, GTPγS (Xu et al., 1999).

i. GRK2 sequesters Gαq and inhibits downstream signalling

G protein-coupled receptor kinases (GRKs) phosphorylate target GPCRs, recruiting arrestins and other proteins involved in receptor desensitization. Additionally, they play a role in a growing number of important cellular functions independently of their action on receptors (Penela et al., 2010). All GRK isoforms possess an amino terminal RH domain, homologous to that present in RGS proteins, and it can differentially interact with members of the Gαq family (Day et al., 2003). Particularly, GRK2 has been reported to interact in a stimulus-dependent manner with Gαq and to display a poor GAP activity towards the G protein compared with RGS proteins (Carman et al., 1999b). It is thought that GRK2 does not predominantly function as an allosteric regulator of Gαq but instead it interacts with the G protein and sequesters it away from receptors and effectors (Ribas et al., 2007). Indeed, the GRK2 RH domain is able to inhibit Gαq-mediated phospholipase C activity both *in vitro* and in intact cell assays, and is more potent a blocker than RGS4 (Carman et al., 1999b; Sallese et al., 2000). These evidences suggest that GRK2 modulatory effect on GPCR signalling can be detected both on the activated receptor as well as on Gαq. GRK2 is able to dampen signalling downstream of several Gq-coupled receptors such as endothelin A and B, thromboxane A2, α1b- adrenergic, M1 and M3 muscarinic cholinergic,

parathyroid hormone, thyrotropin-realising hormone (TRH), 5-hydroxy-tryptamine 2C, metabotropic glutamate receptor-1A (mGluR1A), type 1A angiotensin II and human H1 histamine receptors (Ferguson, 2007). For some of these receptors the sole expression of the RH domain of GRK2 was sufficient to downregulate signalling although it seems generally accepted that GRK2 action has to be dual, both on Gαq and on the GPCR, for efficient attenuation (Ribas et al., 2007).

Cellular responses mediated by Gαq can also be abrogated by GRK2. Insulin stimulates glucose transport through GLUT4 translocation to the plasma membrane by insulin receptor-dependent phosphorylation of Gαq (Imamura & Vollenweider, 1999). This process was shown to be inhibited by the RH domain of GRK2 in 3T3-L1 adipocites and, inversely, the down-regulation of GRK2 increased insulin-mediated GLUT4 translocation (Usui et al., 2004). The sensitization towards insulin pathways in a GRK2-deficient setting has recently been demonstrated to have a major role in insulin resistance and obesity (Garcia-Guerra et al., 2010).

The interaction between Gαq and GRK2 also appears to be regulated by the phosphorylation state of the kinase. We found that phosphorylation of GRK2 by c-Src on tyrosine residues enhances the Gαq/GRK2 interaction, which was consistent with the time-course *in vivo* observed in response to M1 receptor stimulation. Besides, c-Src activation promoted the downregulation of the Gαq/PLCβ signalling pathway in a GRK2 tyrosine phosphorylation-dependent manner (Mariggiò et al., 2006).

GRK2 is also found in lipid rafts through a specific interaction with caveolin mediated by the PH domain of GRK2 (Carman et al., 1999a). Interestingly, caveolin-1/3-bound GRK2 is unable to phosphorylate substrates suggesting that this could be a mechanism for controlling basal GRK activity. The coexistence of GRK2 and Gαq in caveolins has not been reported but it is tempting to speculate that the observed enhancement in Gαq function in caveolae (Sengupta et al., 2008) might be due to a positive scaffolding function of caveolin keeping GRK2 at bay.

The crystal structure of the Gαq-GRK2-Gβγ complex revealed that GRK2 binds to the effector-binding site of Gαq (Tesmer et al., 2005), raising the possibility that GRK2 could indeed be an effector that initiates specific signalling cascades in response to the activation of Gαq. However, despite it has been shown that Gαq can activate GRK2 through PKC (Malhotra et al., 2010), to date there are no reports of a direct involvement of the Gαq/GRK2 complex in triggering downstream signalling.

6. CELLULAR AND PHYSIOLOGICAL FUNCTIONS OF Gαq

It has been estimated that almost 40% of all identified GPCRs rely upon the Gαq/11 family to stimulate inositol lipid signalling (Hubbard & Hepler, 2006). These receptors communicate signals from a number of hormones, neurotransmitters, chemokines, and autocrine and paracrine factors.

The most established role for Gαq is the control of cardiovascular physiopathology, which will be the subject of this section. However, the spectrum of Gαq-mediated cellular functions is very wide and includes a number of other noteworthy examples. Smooth muscle tone is controlled through the action of Gq-coupled receptors such as the angiotensin AT1R, the endothelin ET_A receptor or the α1 adrenoceptor (Wettschureck & Offermanns, 2005). Long-term exposure to angiotensin II results in hypertension and this effect can be ameliorated with the smooth muscle specific overexpression of the carboxyl-terminal end of Gαq, which inhibits receptor coupling (Keys et al., 2002). Additionally, airway smooth muscle responds to Gq-coupled ligands such as acetylcholine to mediate bronchoconstriction (Caulfield, 1993). Gαq also participates in platelet function as shown by the impaired ability of Gαq-/- platelets to aggregate in response to thromboxane A2 (Johnson et al., 1996) which is reflected in the increased bleeding times of Gαq-/- mice (Offermanns et al., 1998). The endocrine system also relies on Gq-coupled receptors: hormone release from the anterior pituitary is controled by hypothalamic releasing hormones, most of which act through Gαq. This is the case for gonadotropin releasing hormone, thyrotropin-releasing hormone and prolactin-releasing hormones (Wettschureck & Offermanns, 2005). Additionally, Gαq is necessary for certain steps of glucose metabolism. 3T3-L1 adipocytes rely on Gαq for basal and insulin-induced translocation of the glucose transporter GLUT4 which is inhibited upon overexpression of RGS2 (Imamura & Vollenweider, 1999). In line with this notion, inhibition of GRK2 increased GLUT4 translocation in response to insulin (Usui et al., 2004). The modulation of synaptic transmission is another example of Gαq involvement in physiology. An important role of Gαq has been described at the parallel fibre-Purkinje cell synapse in the cerebellum, where IP_3-dependent calcium pulses are required for the induction of long-term depression (Miyata et al., 2000). Finally, Gαq signals have a notable proliferative potential and they have transformation properties, but exacerbated Gαq activation results in apoptosis (Fig. I13). After this dual action, Gαq has been referred to as a conditional oncogene (Dhanasekaran et al., 1998). However, mutations in Gq-coupled receptors have been recently demonstrated in multiple human cancers (Kan et al., 2010), and activating mutations in the genes for Gαq family members, Gαq and Gα11, have been identified in approximately 80% of uveal melanomas, where they are now considered to collectively represent the driver oncogene (Raamsdonk et al., 2008; Van Raamsdonk et al., 2010).

a. Gαq function in the heart

The role of Gαq/11 protein family in the heart becomes clear from early stages of embryonic development. Mice lacking both Gαq and Gα11 die at embryonic day 11 as a result of severe thinning of the heart myocardial layer (Offermanns et al., 1998). There likely is some degree of redundancy/compensatory effect since only dual elimination of Gαq and Gα11, but not individual knock-outs, resulted in important phenotypic defects. Gq-coupled GPCR have also been implicated in cardiac growth. Inactivation of 5-HT(2B) gene leads to embryonic and neonatal death caused by heart defects (Nebigil et al., 2000). Hearts from these animals show a lack of trabeculae and ventricular hypoplasia caused by impaired proliferative capacity of myocytes. Also, knock-out mice models for endothelin 1 (ET-1) and its receptors ET_A and ET_B, that couple to Gαq, express a number of cardiac differentiation abnormalities (Giannessi et al., 2001) and the lack of both ET_A and ET_B resulted in mid-gestational heart failure (Yanagisawa et al., 1998).

The onset of cardiac hypertrophy is an adaptative phenomenon in conditions of sustained pressure overload. It is characterised by increased myocyte size and protein content as well as by a characteristic gene expression profile of foetal genes (Rapacciuolo et al., 2001). Despite initially beneficial, hypertrophy has often been identified as a maladaptative process affecting contractile function and increasing the risk of cardiac mortality (Frey & Olson, 2003). Several evidences place Gαq at the centre of hyperthropic pathways in the heart. Agonists such as epinephrine/norepinephrine, angiotensin II, endothelin 1 and serotonin (acting through α1-adrenergic, AT1, ET_A and 5-HT(2B) receptors) have shown to induce hyperthrophy (Wettschureck & Offermanns, 2005). Both cardiac-specific overexpression of the AT1 receptor (Hein et al., 1997) and chronic administration of angiotensin II (Ikeda et al., 2005) can trigger cardiac hypertrophy in mice. Besides, the administration of angiotensin receptor blockers can prevent the development of hypertrophy in mice and in humans (Hunyady & Catt, 2006; Liu et al., 1997). The direct involvement of Gαq-mediated signalling is further stressed by reports showing that cardiac-specific over-expression of Gαq in transgenic mice is enough to trigger cardiac hypertrophy in a similar manner to pressure overload challenges and that it enhances the expression of foetal genes (Liggett, 2006). Finally, conditional inactivation of Gαq/Gα11 in cardiomyocytes leads to a complete absence of hypertrophic response in response to pressure overload (Wettschureck et al., 2001). Taken together these results indicate that Gαq signalling is both necessary and sufficient for the development of cardiac hypertrophy.

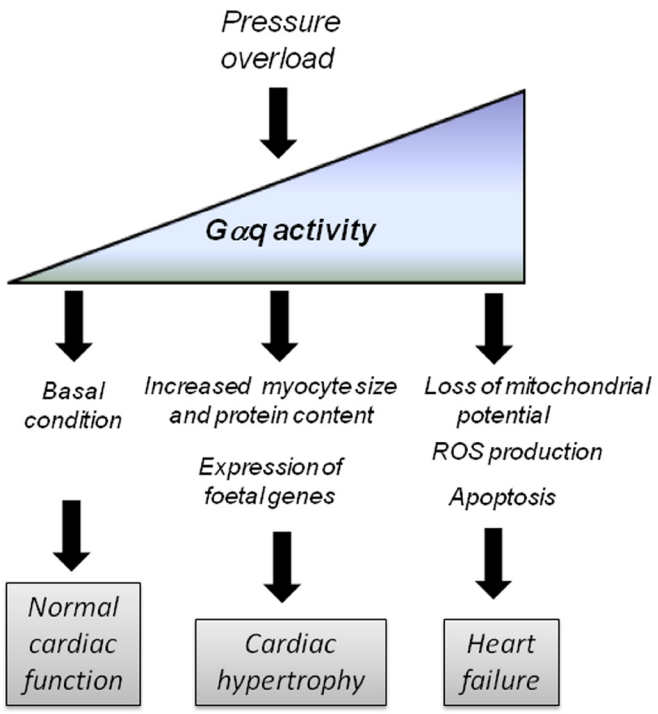

Figure I13. Diversity of Gαq effects in the heart. Activated Gαq signalling contributes to a proliferative/hypertrophic phenotype. Overactivated Gαq signalling initiates apoptotic pathways leading to heart failure.

The development of a cardiac hypertrophy phenotype has been correlated with a higher risk of heart failure (Hunter & Chien, 1999). Interestingly, it has become increasingly clear that both events are tied to the activation/presence of Gαq (Fig. I13). Mice overexpressing Gαq are a well established model of cardiac failure (Fan et al., 2005), which has been linked to the mitochondrial death pathway and to the loss of mitochondrial membrane potential (Adams et al., 2000) as well as to the production of mitochondrial reactive oxygen species (ROS)(Dai et al., 2011). Indeed, whereas the ectopic expression of Gαq in cardiac myocytes recapitulated the hypertrophic phenotype produced by angiotensin II administration, the expression of a constitutively activated mutant of Gαq, which further increased Gαq signalling, produced initial hypertrophy that rapidly progressed to apoptotic cardiomyocyte death. This was also recapitulated by high expression levels of wild-type Gαq. The consequence of cardiomyocyte apoptosis was a transition from compensated hypertrophy to a lethal cardiomyopathy (Adams et al., 1998). These data suggest that moderate levels of Gαq stimulate cardiac hypertrophy signalling whereas high levels of Gαq activation result in cardiomyocyte apoptosis.

Additionally, Gαq also participates in other pathological processes in the heart different from pressure overload-induced hypertrophy such as diabetic cardiomyopathy. Gαq levels and PKC activity were shown to be increased in the streptozotocin-induced diabetic rat heart (Harris et al., 2004).

b. Classical Gαq signalling pathways in the heart

Inhibition of several downstream regulatory proteins has been shown to interfere with Gαq-mediated responses in the heart.

The primary actions of Gq-coupled GPCR in the heart have been tied to the activation of its canonical effector phospholipase Cβ (Fig. I14). A 32-amino acid C-terminal peptide of PLC 1β was sufficient to prevent sarcolemmal membrane targeting of PLC 1β in rat neonatal cardiomyocytes, PLC-mediated IP_3 production and hypertrophic responses elicited by a1AR stimulation (Filtz et al., 2009). PLCβ-initiated pathways can lead to the regulation of the calcineurin/NFAT cascade stimulation and the activation of conventional and novel PKC isoforms.

Inositol phosphate production leads to calcium release into the cytosol where it binds calmodulin (CAM). The Ca^{2++}/CAM complex modulates the function of several proteins. Particularly, calcium-bound CAM will interact with CAMKII and calcineurin, both of which have been shown to play a role in the development of hypertrophy. CAMKII acts differentially through its two isoforms (db, dc) likely controlling different subsets of cardiac proteins and inhibition of CAMKII signalling appears to be a viable mechanism to attenuate hypertrophy and progression to heart failure (Mishra et al., 2010).

DAG promotes PKC activation which leads to the initiation of MAPK signalling as a major route by which Gαq mediates cell growth responses in the heart (Kehat & Molkentin, 2010). Seven different isoforms of PKC (PKCα, -β1, -β2, -δ, -ε, -λ and -ζ) are expressed in most adult mammalian myocardia (Dorn & Force, 2005). PKC activation regulates contractility and hypertrophy in cultured cardiomyocytes but mouse models deficient in individual isoforms do no present a cardiac phenotype, a phenomenon likely due to functional compensation. Most notably, PLCα overexpression promotes hypertrophy and its inhibition blocks heart failure both in cultured myocytes and in mice (Braz et al., 2004). Also, the normalisation of PLCα protein levels in Gαq-overexpressing hypertrophy models (where PLCα is uregulated) improves contractility, which points to an additional role of PLCα on systolic and diastolic function by β-adrenergic receptor desensitisation (Hahn et al., 2003). Transgene expression of PLCβ in adults caused ventricular hypertrophy that was associated with impaired diastolic relaxation, whereas expression in newborns caused sudden death associated with marked abnormalities in the regulation of intracellular calcium (Bowman et al., 1997). Additionally, PLCε pharmacological inhibition during development causes embryo lethality due to cardiac hypoplasia but enhancement on its activity in the adult heart (by promoting plasma membrane translocation) reduces cardiac hypertrophy (Mochly-Rosen et al., 2000; Wu et al., 2000). These data support the notion

that PLCε signalling is a compensatory event in hypertrophy, rather than a pathological event, and suggest the therapeutic potential of PLCε enhancement to improve cardiac insufficiency.

MAPKs are the primary downstream effectors in cardiovascular function acting as signalling switches between apoptosis and adaptive hypertrophy. For example, in mice, p38 mitogen-activated protein kinases are strongly activated by pressure overload, and upstream kinases that specifically activate p38 cause the growth of cultured myocytes but this activation is also accompanied by an increase in the rate of apoptosis (Wang et al., 1998a). Specific activation of the JNK pathway in cardiac myocytes induced characteristic features of hypertrophy but, in contrast, co-activation of both JNK and p38 led to an induction of cytopathic responses and suppression of hypertrophic programmes (Wang et al., 1998b). Similarly, the specific implication of ERK1/2 is diverse. Whereas it is known to contribute to concentric cardiomyocyte hypertrophy, associated with increased cardiomyocyte width due to new sarcomeres synthesis and ERK1/2 autophosphorylation (Lorenz et al., 2009), the inhibition of ERK1/2 signalling increases eccentric hypertrophy, associated with augmented cardiomyocyte length (Kehat et al., 2011). Besides, the inhibition of several ERK1/2 downstream targets, including mitogen and stress-activated kinase 1 and MAP kinase-interacting kinase 1, has also been shown to reduce hypertrophy in cardiomyocytes (Markou et al., 2009; Spruill & McDermott, 2006) thus suggesting that the ambiguous effect of ERK1/2 in hypertrophy could be due to the differential action of alternative downstream cascades.

Despite the relevance of PLCβ as a primary effector of Gαq actions in the heart, there is evidence that unveils a lack of correlation between PLCβ activation and the hypertrophic phenotype in the Gαq-overexpressing mouse model (Mende et al., 2001). Additionally, several reports have shown MAPK activation independently of PLCβ activity in a number of cellular contexts (Berts et al., 1999; Coso et al., 1995; Heasley & Storey, 1996; Peavy et al., 2001; Seo et al., 2000). Thus, alternative players are thought to be mediating part of the essential function of Gαq in the heart. Indeed, we recently described the occurrence of the Gαq-PKCζ-ERK5 signalling axis as an important mediator of hypertrophic genetic programmes in the heart (see next section and results).

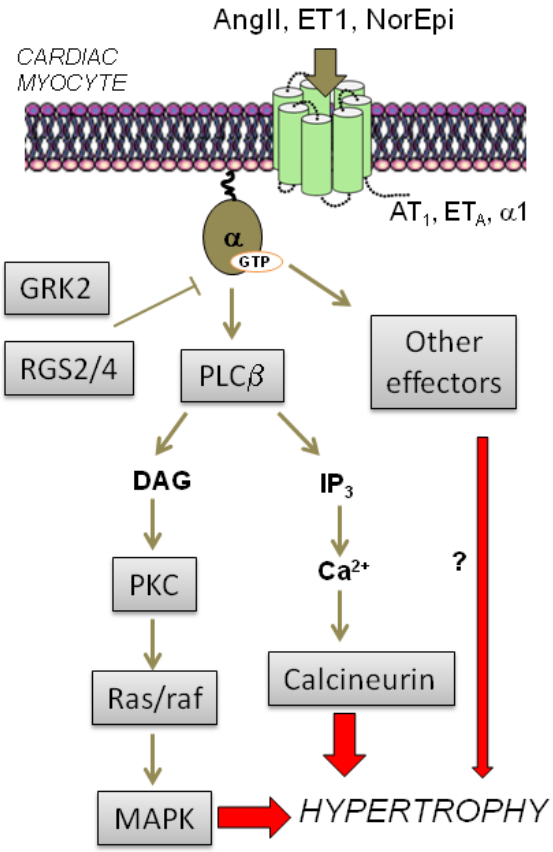

Figure I14. Classical Gαq hypertrophic pathways in the heart. Gαq hypertrophic responses in the heart are thought to be mediated by PLCβ and they can be calcium-dependent or independent. The first lead to calcineurin activation and the latter lead to MAPK activation. All these responses are negatively regulated by GRK2 and RGS proteins. Additional effectors remain to be elucidated.

c. Emerging roles of PKCζ and ERK5 in the heart

PKCζ and ERK5, recently described components of a novel Gαq-initiated signalling axis (see section 5e), have also been involved in different cardiovascular functions.

As mentioned above, the role of MAPK signalling in Gαq-promoted hypertrophy has been extensively characterised and usually associated to the activation of the ERK1/2 pathway. However, also ERK5 plays an important role in heart function that is clear since early stages of development. Deletion of either ERK5 or MEK5 is embryonic lethal at approximately E10 due to an underdeveloped myocardium, disorganization of the trabeculae, and vascular defects (Hayashi & Lee, 2004). However, at least part of the lethal phenotype in development cannot be ascribed to the function of ERK5 in cardiomyocytes since targeted deletion of the kinase in this cell type showed normal development, whereas specific defect in endothelial ERK5 recapitulated the phenotype from the global knockout (Hayashi & Lee, 2004; Hayashi, 2004). However, the role of ERK5 in cardiovascular function has been extensively demonstrated. It has been reported that Angiotensin II promotes ERK5 activation in mice myocardium (Ikeda et al., 2005), that cardiac-specific overexpression

of either upstream activators (MEK5)(Nicol et al., 2001) or downstream targets (MEF2A and C)(Xu et al., 2006) of ERK5 induces cardiac hypertrophy in mice, and that the activity of ERK5 is increased during left ventricular hypertrophy (Kacimi & Gerdes, 2003; Takeishi et al., 2001), whereas targeted deletion of ERK5 in cardiomyocytes attenuates the hypertrophic response in the heart (Kimura et al., 2010).

Also PKCζ has been shown as an increasingly important molecule in cardiac function. PKCζ has been reported to augment atrial natriuretic factor in ventricular cardiomyocytes (Decock et al., 1994) and more recently to be involved in cardiac sarcomeric protein phosphorylation (Wu & Solaro, 2007), which can be a relevant process during mechanical heart stress (Borges et al., 2008). Additionally, the protein kinase C (PKC)-interacting cousin of thioredoxin (PICOT) exerts two distinct effects, through previously unknown mechanisms, in the heart: it inhibits cardiac hypertrophy and enhances cardiac contractility (Jeong et al., 2006). Recently it was reported that PICOT affected cardiac contractility by inhibiting PKCζ and leading to higher calcium release and sensitivity in cardiomyocytes (Oh et al., 2012). This phenotype can be recapitulated by the PKCζ pseudosubstrate specific inhibitor. Besides, data in isolated adult cardiac myocytes demonstrate that stimulation of a Gq-coupled GPCR, the thromboxane TxA2 receptor, directly induces cardiac myocyte apoptosis which can be ascribed, to some degree, to a reduction in Akt activity mediated by PKCζ (Shizukuda & Buttrick, 2002). As reported for ERK5, PKCζ is also involved in endothelial function, which indirectly affects cardiovascular homeostasis. Indeed, pharmacological inhibition of PKCζ was shown to exert cardioprotective effects in ischemia-reperfusion injury due to a decrease in nitric oxide released by the endothelium (Phillipson et al., 2005).

Taken together, these data put forward PKCζ and ERK5 as main players in cardiovascular function. As detailed in section 5e we have recently described the activation of ERK5 by Gq-coupled GPCR involving PKCζ in epithelial cells (García-Hoz et al., 2010). Thus, we investigated the occurrence of this novel signalling axis in cardiac cells. The first part of this research project is included in the Results section so it will not be explained in detail here. Briefly, we established that ERK5 is activated by the Gq-coupled ligand angiotensin II in both adult and neonatal cardiac myocytes and in neonatal fibroblasts. This pathway was dependent upon PKCζ activity or presence as demonstrated by the abrogated activation of ERK5 upon PKCζ knockdown or pharmacological inhibition. Additionally, we established the occurrence of the pathway in cardiac tissue from angiotensin II-treated wild-type mice whereas this could not be detected in PKCζ-/- mice (see Results section). In order to assess the relevance of the pathway *in vivo* we induced cardiac hypertrophy in PKCζ KO mice and control littermates through chronic angiotensin infusion for 14 days. A global microarray was performed to determine differential expression patterns between

AngII or PBS-treated mice of the two different breeds, and compared it with published results for mouse models of hypertrophy (TAC or AT1-overexpressing mice). The 1,536 probesets that achieved statistically significant criteria for differential expression were listed in two groups as either overexpressed (group I) or underexpressed (group II) in hypertrophy, compared to controls. According to this classification, the models for hypertrophy segregated differently with respect to control animals, which served us to define a hypertrophic expression pattern. In our experimental conditions, angiotensin-treated wt mice showed a clear hypertrophic expression pattern whereas the expression profile of angiotensin-treated PKCζ KO mice segregated with control animals. Thus, we reported that the presence of PKCζ was necessary to display a hypertrophic genetic profile in response to angiotensin II. We found that hypertrophic markers such as ANP (Nppa) or type I collagen (Col1α2) were not overexpressed in angiotensin II-treated PKC-deficient mice compared to wild-type. This was further confirmed by quantitative real time PCR. Additionally, several downstream targets of ERK5 (ets-1, ccna2, bcl2), and ERK5 itself, were upregulated in control as opposed to PKCζ KO animals. Finally, several physiological markers of hypertrophy were found to be consistent with the genetic profile data. Plasma ANP was increased in control but not in PKCζKO animals. Similarly, heart weight/body weight or heart weight/tibial length ratios were increased in controls compared to PKCζ KO. Also, an electrocardiographic analysis showed an increase in QRS and QT components (which has been correlated to left ventricular hypertrophy (Salles et al., 2005)) in control animals whereas this was not observed in PKCζ-/- mice. Taken together, these results show that PKCζ is essential for the promotion of a hypertrophic phenotype upon angiotensin II challenge (García-Hoz et al., 2012) (Fig. I15).

d. Gαq signalling regulation in the heart

The regulation of Gαq function in the heart is an important process for limiting signal duration and amplitude. As expected, the two main families of G protein regulators, GRKs and RGS proteins, have been involved in cardiovascular physiopathology. However, the outcome of their regulatory functions is not at all straightforward, especially in the case of GRK2 that plays numerous roles besides the downregulation of Gαq signalling (Ribas et al., 2007).

The overexpression of a dominant-negative Gαq peptide or the Gαq negative regulator RGS4 in murine heart, renders resistance to pressure overload-triggered hypertrophy (Rogers et al., 1999; Rohini et al., 2010). However, as mentioned above, the function of Gαq in the heart is dual since it can mediate potent proliferative signals, which results in hypertrophy, and also pro-apoptotic signals leading to heart failure. Thus, RGS4 overexpression not only abrogates hypertrophy but also results in rapid cardiac decompensation, depressed systolic function, and increased mortality (Rogers et al., 1999). However, another

report for the heart-specific overexpression of RGS4 in mice found that it provides protection against different models of diabetic cardiomyopathy and, conversely, expression of a RGS-resistant Gαq caused sensitization towards this type of pathology (Yang et al., 1999).

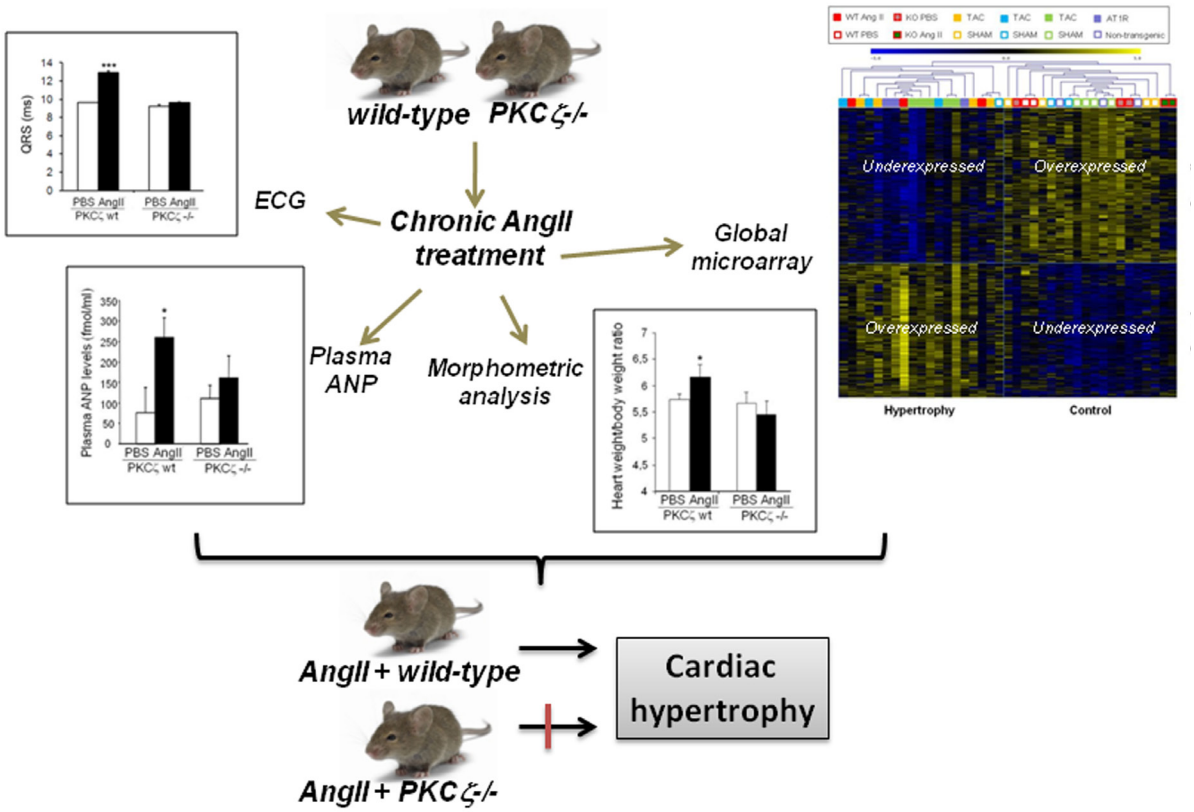

Figure I15. The Gαq/PKCζ/ERK5 is involved in heart hypertrophy. Wild-type or PKCζ-/- mice were subjected to Angiotensin II chronic stimulation to detect changes in hypertrophy development. Hypertrophy parameters such as electrocardiogram, plasma ANP, morphometric or gene expression analyses indicate that PKCζ-/- do not develop AngII-induced hypertrophy(García-Hoz et al., 2012).

GRK2 is a multifunctional protein that not only acts as a negative regulator of GPCR signalling but also has been pointed as an important signalling hub that initiates a large number of pathways (Penela et al., 2010). In order to study the role of GRK2 as Gαq regulator in isolation with respect to other functions, the overexpression of the RH domain of the kinase has demonstrated to be a fruitful approach. It is in this domain where the Gαq binding/sequestering function resides (section 5i) and its overexpression has been shown to interfere with α1 adrenoceptor-induced cardiomyocyte hypertrophy (Maruyama, 2002). This is yet another proof of concept for the strict requirement of Gαq function for the promotion of hypertrophy. However, it has been described that the effect of GRK2 on Gαq activation in the heart coexists with an important role in the desensitisation of β adrenergic receptors, Gs-coupled GPCRs that are main mediators of cardiac contractility.

Hemizygous GRK2 mice expressing 50% less protein than control littermates are hyperresponsive to catecholamines and present increased cardiac contractility and function. The opposite is observed in transgenic GRK2-overexpressing mice (Koch et al., 1998). Additionally, increased left ventricular GRK2 mRNA and activity were reported in patients with ischemic or idiopathic dilated cardiomyopathy, cardiac ischemia, volume overload and left ventricular hypertrophy (Penela et al., 2006). These increased levels might reflect a compensating mechanism in pathologies with predominance of β adrenergic and Gαq-mediated signalling. Finally, the overexpression of GRK2-C terminal domain rescues cardiac dysfunction and improves survival in many different mouse models of heart failure, although it does not prevent cardiac deterioration in Gαq transgenic mice despite the fact that normal βAR signalling was restored (Penela et al., 2010). These results suggest that the negative role in Gαq-driven responses is not mediated by receptor desensitisation by the C terminal domain, as it is the case for βAR regulation, but occurs more likely through Gαq sequestering. Moreover, it is important to highlight that GRK2 displays an array of functions independently of GPCR signalling through various different protein-protein interactions. Thus, the possibility that the positive therapeutic outcomes of the use of GRK2ct might be partly due to interference with binding partners such as PI3K/Akt, tubulin, ezrin or GIT1 cannot be ruled out.

7. CONCLUDING REMARKS

In the light of novel findings on the large plethora of Gαq-interacting proteins the scientific community is now able to define a much more detailed map of the different effectors involved in each Gαq-initiated pathway and in the subsequent cellular events. Thus, a careful inspection of novel pathways will provide the basis of a deep understanding on Gαq function. The performance of this approach to the interaction of Gαq with the novel effector PKCζ has been the conceptual foundation for this thesis.

II. OBJECTIVES

1. Investigate the occurrence of the Gαq/PKCζ/ERK5 axis in the cardiovascular system.

2. Establish the relative contribution of Gαq and β-arrestin routes towards the activation of ERK5 by Gq-coupled GPCRs.

3. Determine the occurrence and specificity of the Gαq/PKCζ complex in living cells

4. Identify the interaction surfaces involved in the formation of the Gαq/PKCζ complex

5. Establish the requirement of Gαq/PKCζ association for the activation of the ERK5 pathway.

6. Identify the regulation mechanisms exerted by GRK2 on the Gαq/PKCζ complex as well as on ERK5 activation.

7. Study the activation mechanisms of PKCζ by Gq-coupled GPCRs.

8. Explore the potential role of the Gαq/PKCζ axis in other cellular processes such as proliferation and apoptosis.

III. MATERIALS & METHODS

1. MATERIALS

a. Buffers and solutions

Buffer	Composition	Comments
DNA sample buffer 6x	18% (v/v) glycerol 0.15% (w/v) bromophenol blue 0.15% (w/v) xylencyanol 15mM EDTA	
ERK5 buffer	50 mM Tris-HCl pH 7.5 150 mM NaCl 1mM NP-40 0.25% deoxycholate 1 mM EGTA pH 8.0 1 mM NaF	stored at 4°C
	0.1 µM PMSF 0.5 µM Benzamidin 1 µg/µl Aprotinin 0.1 µM $NaVO_3$	added fresh

Hanks medium	137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$ 0.33 mM Na_2HPO_4 0.44 mM KH_2PO_4 1.0 mM $MgCl_2$ 0.15 mM Tris-HCl 1.0 mM sodium butirate, pH 7.4	
Laemmli polyacrylamide gel (6-12%)	20-40% (v/v) acrylamide/ N,N'methylen- bisacrylamide 30:0.8 (w/v) 0.375 M Tris-HCl pH 8.9 0.1% SDS	
Laemmli running buffer (10x)	0.25 M Tris base 2.0 M glycine 1% SDS	
LB broth	1% (w/v) bacto-tryptone 0.5% (w/v) bacto-yeast extract 1% (w/v) NaCl (171 mM)	autoclaved
LB-agar	LB broth + 1.5% (w/v) agar	autoclaved
PBS (1x)	137 mM NaCl (0.8% w/v) 2.7 mM KCl (0.02% w/v) 10 mM Na_2HPO_4 (0.144% w/v) 1.75 mM KH_2PO_4 (0.024% w/v) pH 7.4	autoclaved
Ponceau staining solution	0.2% Ponceau-S in 3.0% TCA	
RIPA buffer (2x)	0.08 ml 0.5 M EDTA pH 8.0 0.72 ml 37% formaldehyde 2.00 ml 100% glycerol 3.084 ml formamide 4.00 ml 10x MOPS buffer 0.016 ml bromophenol blue RNase free water to 10 ml	store at 4°C for less than 3 months
	0.1 µM PMSF 0.5 µM Benzamidin 1 µg/µl Aprotinin 0.1 µM $NaVO_3$	Added fresh
Sorting buffer	PBS 5mM EDTA 25mM HEPES pH 7 supplemented with 2% FBS	stored at -20°C

Sample buffer (4x)	80 mM Tris-HCl pH 6.8 2% SDS 10% glycerol 0.2% (w/v) bromophenol blue 50 ml/ml (v/v) 2-mercaptoethanol	stored at 4°C
TAE buffer (1x)	40 mM Tris-acetate pH 7.6 1 mM EDTA	autoclaved
TBS (10x)	0.1 M Tris base 1.5 M NaCl pH 7.4 with HCl conc.	
TBS-Tween (10x)	0.1 M Tris base 1.5 M NaCl 0.5% Tween-20 pH 8.0 with HCl conc.	
Tyrode solution	37 mM NaCl 2.7 mM KCl 1 mM $MgCl_2$ 1.8 mM $CaCl_2$ 0.2 mM Na_2HPO_4 12 mM $NaHCO_3$ 5.5 mM D-glucose	
Transfer buffer (10x)	0.2 M Tris base 1.5 M glycine 20% methanol (v/v)	

b. Oligonucleotides

All oligonucleotides for mutagenesis were purchased from Sigma-Aldrich Spain whereas those used for cloning were purchased from Integrated DNA Technologies (IDT), Quebec, Canada.

Primer name	5´-3´ sequence
Gαq E234A FW	CT CCA TCA TGT TTC TAG TAG CGC TTA GCG CGT ATG ATC AAG TTC TTG TGG A
Gαq E234A REV	T GTC TGA CTC CAC AAG AAC TTG ATC ATA CGC GCT AAG CGC TAC TAG AAA CA
Gαq D236A FW	GTA GCG CTT AGT GAA TAT GCG CAA GTT CTC GTG GAG TCA
Gαq D236A REV	TGA CTC CAC GAG AAC TTG CGC ATA TTC ACT AAG CGC TAC
Gαq D243A FW	CAA GTT CTC GTG GAG TCA GCG AAT GAG AAC CGA ATG GAG

Gαq D243A REV	CTC CAT TCG GTT CTC ATT CGC TGA CTC CAC GAG AAC TTG
Gαq N244A FW	GTT CTC GTG GAG TCA GAC GCG GAG AAC CGA ATG GAG GAA
Gαq N244A REV	TTC CTC CAT TCG GTT CTC CGC GTC TGA CTC CAC GAG AAC
Gαq E245A FW	T CAA GTT CTT GTG GAG TCA GAC AAT GCG AAC CGC ATG GAG GAG AG
Gαq E245A REV	C TTT GCT CTC CTC CAT GCG GTT CGC ATT GTC TGA CTC CAC AAG AA
Gαq E249A FW	GAC AAT GAG AAC CGA ATG GCG GAA AGC AAG GCT CTC TTT
Gαq E249A REV	AAA GAG AGC CTT GCT TTC CGC CAT TCG GTT CTC ATT GTC
Gαq S251A FW	GAG AAC CGA ATG GAG GAA GCG AAG GCT CTC TTT AGA ACA
Gαq S251A REV	TGT TCT AAA GAG AGC CTT CGC TTC CTC CAT TCG GTT CTC
Gαq E245A/S251A FW	GAG TCA GAC AAT GCG AAC CGA ATG GAG GAA GCG AAG GCT CTC TTT
Gαq E245A/S251A REV	AAA GAG AGC CTT CGC TTC CTC CAT TCG GTT CGC ATT GTC TGA CTC
Gαq E241/D243/ E245 FW	CAA GTT CTT GTG GCG TCA GCG AAT GCG AAC CGA ATG GAG
Gαq E241/D243/ E245 REV	CTC CAT TCG GTT CGC ATT CGC TGA CGC CAC AAG AAC TTG
Gαq E243/E244/ E249 FW	AAT GCG AAC CGC ATG GCG GCG AGC AAA GCA CTC TTT
Gαq E243/E244/ E249 REV	AAA GAG TGC TTT GCT CGC CGC CAT GCG GTT CGC ATT
PKCζ R15A FW	ATG GAC CGG AGC GGC GGC GCG GTC CGT CTG AAG GCG CAC
PKCζ R15A REV	GTG CGC CTT CAG ACG GAC CGC GCC GCC GCT CCG GTC CAT
PKCζ R17A FW	CGG AGC GGC GGC CGC GTC GCG CTG AAG GCG CAC TAC GGC
PKCζ R17A REV	GCC GTA GTG CGC CTT CAG CGC GAC CGC GCC GCC GCT CCG
PKCζ K19A FW	GGC GGC CGC GTC CGT CTG GCG GCG CAC TAC GGC GGG GAC
PKCζ K19A REV	GTC CCC GCC GTA GTG CGC CGC CAG ACG GAC GCG GCC GCC

PKCζ H21A FW	CGC GTC CGT CTG AAG GCG GCG TAC GGC GGG GAC ATC CTG
PKCζ H21A REV	CAG GAT GTC CCC GCC GTA CGC CGC CTT CAG ACG GAC GCG
PKCζ I26A FW	GCG CAC TAC GGC GGG GAC GCG CTG ATT ACC AGC GTG GAC
PKCζ I26A REV	GTC CAC GCT GGT AAT CAG CGC GTC CCC GCC GTA GTG CGC
PKCζ I92A FW	GGC AGG GAC GAA GTG CTC GCG ATC CAC GTT TTC CCA AGC
PKCζ I92A REV	GCT TGG GAA AAC GTG GAT CGC GAG CAC TTC GTC CCT GCC
PKCζ R15A/K19A FW	GAC CGG AGC GGC GGC GCG GTC CGT CTG GCG GCG CAC TAC GGC GGG
PKCζ R15A/K19A REV	CCC GCC GTA GTG CGC CGC CAG ACG GAC CGC GCC GCC GCT CCG GTC
PKCζ R17A/K19A FW	CGG AGC GGC GGC CGC GTC GCG CTG GCG GCG CAC TAC GGC GGG GAC
PKCζ R17A/K19A REV	GTC CCC GCC GTA GTG CGC CGC CAG GCG GAC GCG GCC GCC GCT CCG
PKCζ I26A/K19A FW	GTC CGT CTG GCG GCG CAC TAC GGC GGG GAC GCG CTG ATT ACC
PKCζ I26A/K19A REV	GGT AAT CAG CGC GTC CCC GCC GTA GTG CGC CGC CAG ACG GAC
Gαq Q209L FW	GAT GTA GGG GGC CTA AGG TCA GAG AGA
Gαq Q209L REV	TCT CTC TGA CCT TAG GCC CCC TAC ATC
Gαq R183C FW	CAA GAC GTC CTT AGA GTT TGT GTC CCC ACT ACA GGG ATC
Gαq R183C REV	GAT CCC TGT AGT GGG GAC ACA AAC TCT AAG GAC GTC TTG
Gαq Y261F FW	ACA ATT ATC ACC TTC CCC TGG TTC CAG
Gαq Y261F REV	CTG GAA CCA GGG GAA GGT GAT AAT TGT
PKCζ-V1 (INSERT) FW	CAC AGT GGC GGC CGC ACC ATG CCC AGC AGG ACC GAC CC
PKCζ-V1 (INSERT) REV	GAG CCA CCG CCA CCA TCG ATC ACG GAC TCC TCA GCA GAC A
PKCζ-V1 (PLASMID) FW	TGT CTG CTG AGG AGT CCG TGA TCG ATG GTG GCG GTG GCT C
PKCζ-V1 (PLASMID) REV	GGG TCG GTC CTG CTG GGC ATG GTG CGG CCG CCA CTG TG
V1-PKCζ (INSERT) FW	GAG GTG GTG GGT CCT CCG GAA TGC CCA GCA GGA CCG ACC C

V1-PKCζ (INSERT) REV	GTT TAA ACG GGC CCT CTA GAT CAC ACG GAC TCC TCA GCA G
V1-PKCζ (PLASMID) FW	CTG CTG AGG AGT CCG TGT GAT CTA GAG GGC CCG TTT AAA C
V1-PKCζ (PLASMID) REV	GGG TCG GTC CTG CTG GGC ATT CCG GAG GAC CCA CCA CCT C
Gαq-V1 (INSERT) FW	CAC AGT GGC GGC CGC ACC ATG ACT CTG GAG TCC ATC ATG GCG
Gαq-V1 (INSERT) REV	GAG CCA CCG CCA CCA TCG ATG ACC AGA TTG TAC TCC TTC AGG TTC A
Gαq-V1 (PLASMID) FW	TGA ACC TGA AGG AGT ACA ATC TGG TCA TCG ATG GTG GCG GTG GCT C
Gαq-V1 (PLASMID) REV	CGC CAT GAT GGA CTC CAG AGT CAT GGT GCG GCC GCC ACT GTG
V1-Gαq (INSERT) FW	GAG GTG GTG GGT CCT CCG GAA TGA CTC TGG AGT CCA TCA TGG C
V1-Gαq (INSERT) REV	GTT TAA ACG GGC CCT CTA GAT TAG ACC AGA TTG TAC TCC TTC AGG TTC
V1-Gαq (PLASMID) FW	GAA CCT GAA GGA GTA CAA TCT GGT CTA ATC TAG AGG GCC CGT TTA AAC
V1-Gαq (PLASMID) REV	GCC ATG ATG GAC TCC AGA GTC ATT CCG GAG GAC CCA CCA CCT C
GαqSEC	CAG AAT GGT CGA TGT AGG GGG CCA AAG
GαqSEC II	TGA TCC TGG AAT CCA GGA ATG CTA TGA TAG ACG ACG AGA

c. DNA enzymes

Enzyme	**Supplier**
Pfu ultra	Stratagene
Platinum Taq	Invitrogen
Taq polymerase	Biolabs
T4 DNA ligase	Invitrogen
*Bspe*I	NEB
*Cla*I	NEB
*Nar*I	NEB
*Nhe*I	Fermentas
*Not*I	NEB
*PspOM*I	NEB
*Xba*I	Fermentas

d. Primary antibodies

Primary antibodies were used at concentrations ranging 1:500-1:1000 in TBS-Tween 1x, except from anti-phosphoERK5 antibodies when used for detection of endogenous phosphorylation that were freshly made in 3% phosphoblocker (Cell Biolabs).

Antigen	Reference	Supplier	Species
Actin	sc-1616	Santa cruz	Goat
Akt	9272	Cell Signaling	Rabbit
Akt	9272	New England Biolabs	Rabbit
α-Tubulin	DM1A	Sigma	Mouse
Erk1	(C-16): sc-93	Santa cruz	Rabbit
Erk2	(c-14): sc-154	Santa cruz	Rabbit
ERK5	3372	Cell Signaling	Rabbit
ERK5	Q13164	Millipore	Rabbit
ETS-1 (C-20)	sc-350	Santa cruz	Rabbit
FLAG M1	F3040	Sigma	Mouse
GAPDH	ab8245	Abcam	Mouse
Gαq (e-17)	sc-393	Santa cruz	Rabbit
Gαq/11 (C-19)	sc-392	Santa cruz	Rabbit
GRK2 (C-15)	sc-562	Santa cruz	Rabbit
GRK2/3 (b-ARK 1/2)	05-465	Millipore	Mouse
GRK2 PF1		Generated in the laboratory	Rabbit
GST	(Z-5): sc-459	Santa cruz	Rabbit
HA Y-11	sc-805	Santa cruz	Rabbit
HA(F-7)	sc-7392	Santa cruz	Rabbit
HA(F-7)-agarose	sc-7392-AC	Santa cruz	Mouse
HA-11	afc-101p	Covance	Mouse
MEF2c (C-17)	sc-13268	Santa cruz	Goat
MEK5	ab-24828	Abcam	Rabbit
p-Akt (S473)	9271	Cell Signaling	Rabbit
p-Akt (T308)	9275	Cell Signalling	Rabbit
p-Erk1-2 (T202/T204)	9101	Cell Signaling	Rabbit
p-ERK5 (T218/Y220)	44612g	Invitrogen	Rabbit
PKCζ (C-20)	sc-216	Santa cruz	Rabbit
PKCζ (C24E6)	9368	Cell signalling	Rabbit
PKCζ (H-1)	sc-17781	Santa cruz	Mouse
p-MEF2c (S387)	sc-13920-R	Santa cruz	Rabbit
p-PKCζ (Thr-410)	sc-12894-R	Santa cruz	Rabbit
p-PKCζ(T560)	ab62372	Abcam	Rabbit

e. Secondary antibodies

Name, antigen	Company	Dilution
Alexa Fluor 680, goat anti-mouse IgG	Molecular probes	1 mg/ml
IRDyeTM 800 CW, donkey anti-mouse IgG	Rockland	0.5 mg/ml
Alexa Fluor 680, goat anti-rabbit IgG	Molecular probes	1 mg/ml
IRDyeTM 800 CW, goat anti-rabbit IgG	Rockland	0.5 mg/ml
Alexa Fluor 680, donkey anti-goat IgG	Molecular probes	1 mg/ml
Soluble rabbit peroxidase-anti-mouse IgG	Nordic immunology	1/50000
Soluble mouse peroxidase-anti-rabbit IgG	Nordic immunology	1/50000

f. Plasmids

cDNA	Species	Vector	Origin
ERK5	Human	pcDNA3.1	Dr J. Moscat (Sanford-Burnham, La Jolla, USA)
Gαq wt	Mouse	pCIS	Dr A.Aragay (IBMB, Spain)
Gαq R183C	Mouse	pCIS	Dr A.Aragay (IBMB, Spain)
Gαq Q209L	Mouse	pCIS	Dr A.Aragay (IBMB, Spain)
Gαq T257E	Mouse	pCMV	Dr T. Kozasa
Gαq W263D	Mouse	pCMV	Dr T. Kozasa
Gαq Y261F	Mouse	pCMV	Dr T. Kozasa
Gαq Y261F	Mouse	pcDNA3.1	Generated as part of this thesis
Gαq R183C-Y261F	Mouse	pcDNA3.1	Generated as part of this thesis
Gαq E234A	Mouse	pCIS	Generated as part of this thesis
Gαq D236A	Mouse	pCIS	Generated as part of this thesis
Gαq D243A	Mouse	pCIS	Generated as part of this thesis
Gαq N244A	Mouse	pCIS	Generated as part of this thesis
Gαq E245A	Mouse	pCIS	Generated as part of this thesis
Gαq E245A	Mouse	pCIS	Generated as part of this thesis
Gαq E249A	Mouse	pCIS	Generated as part of this thesis
Gαq S251A	Mouse	pCIS	Generated as part of this thesis
Gαq E234/245-AA	Mouse	pCIS	Generated as part of this thesis
Gαq E245/S251-AA	Mouse	pCIS	Generated as part of this thesis
Gαq E234/E241/D243/E245-AAAA	Mouse	pCIS	Generated as part of this thesis

Gαq E234/E243/E244/E249-AAAA	Mouse	pCIS	Generated as part of this thesis
Gαq R83C-E234/245-AA	Mouse	pCIS	Generated as part of this thesis
Gαq wt (inducible)	Mouse	pcDNA-4TO	Dr A.Aragay (IBMB, Spain)
Gαq R183C (inducible)	Mouse	pcDNA-4TO	Dr A.Aragay (IBMB, Spain)
Gαq Q209L (inducible)	Mouse	pcDNA-4TO	Generated as part of this thesis
Gαq R83C-E234/245-AA (inducible)	Mouse	pcDNA-4TO	Generated as part of this thesis
Gαq Q209L-E234/245-AA (inducible)	Mouse	pcDNA-4TO	Generated as part of this thesis
Gαq-Venus YFP[F1]	Mouse	pcDNA3.1	Generated as part of this thesis
Venus YFP[F1]-Gαq	Mouse	pcDNA3.1	Generated as part of this thesis
Gαq-Venus YFP[F2]	Mouse	pcDNA3.1	Generated as part of this thesis
Venus YFP[F2]-Gαq	Mouse	pcDNA3.1	Generated as part of this thesis
Gα11-Venus YFP[F1]	Human	pcDNA3.1	Dr SW Michnick (Universite de Montreal, Canada)
Gαi1-Venus YFP[F1]	Human	pcDNA3.1	Dr SW Michnick (Universite de Montreal, Canada)
IQ23 (Gαi-ct Gαq)	Human	pcDNA1	Dr C. Berlot (Yale University, USA)
QI4 (Gαq-ct Gαi)	Human	pcDNA1	Dr C. Berlot (Yale University, USA)
PKCζ-HA wt	Rat	pcDNA3.1	Dr J. Moscat (Sanford-Burnham, La Jolla, USA)
PKCζ-HA R15A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA R17A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA K19A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA H21A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA I26A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA I92A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA R15A/K19A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA R17A/K19A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA I26A/K19A	Rat	pcDNA3.1	Generated as part of this thesis
PKCζ-HA PB1	Rat	pcDNA3.1	Dr J. Moscat (Sanford-Burnham, La Jolla, USA)
PKCζ-HA KD	Rat	pcDNA3.1	Dr J. Moscat (Sanford-Burnham, La Jolla, USA)
PKCζ-Venus YFP[F1]	Rat	pcDNA3.1	Dr SW Michnick (Universite de Montreal, Canada)
PKCζ-Venus YFP[F2]	Rat	pcDNA3.1	Generated as part of this thesis

Venus YFP[F2]-PKCζ	Rat	pcDNA3.1	Generated as part of this thesis
PLCβ-YFP[F1]	Human	pcDNA3.1	Dr SW Michnick (Universite de Montreal, Canada)
GRK2	Human	pcDNA3.1	Dr J Benovic (The Kimmel Cancer Center, USA)
GRK2 K220R	Human	pcDNA3.1	Dr J Benovic (The Kimmel Cancer Center, USA)
GRK2 D110A	Human	pcDNA3.1	Dr JG Tesmer (University of Michigan, USA)
GRK2-RGS 2-187	Human	pcDNA3.1	Generated in the laboratory
MEK5 (GST)	Human	pcDNA3.1	Dr J. Moscat (Sanford-Burnham, La Jolla, USA)

g. Agonists and inhibitors

Name	Function	Concentration	Time
Acetycholine	Muscarinc receptor natural agonist (M1, M2, M3)	0.01μM-100mM	Timecourse
Carbachol	Muscarinc receptor non-hydrolysable agonist (M1, M2, M3)	10μM	Timecourse
AngII	AT1 and AT2 receptor natural agonist	100nM	Timecourse
SII (Sar1-Ile4-Ile8 Ang II)	β-arrestin-biased Angiotensin agonist	30mM	Timecourse
Pertussis toxin	Gαi protein family inhibitor	100ng/ml	16h
PKCζ myristoylated pseudosubstrate	PKCζ inhibitor	10μM	30min
U73122	PLC inhibitor	10μM	30min

h. Cell lines

Common cell lines	Origin
HELA	ATCC
HEK293	ATCC
COS-7	ATCC

Stable cell lines	Origin
CHO M1	Dr AB Tobin (University of Leicester, UK)
CHO M3	Dr AB Tobin (University of Leicester, UK)
CHO M3 SASS	Dr AB Tobin (University of Leicester, UK)
CHO M1 flip in	Dr AB Tobin (University of Leicester, UK)
CHO M1 flip in phosphodef	Dr AB Tobin (University of Leicester, UK)
CHO M3 flip in	Dr AB Tobin (University of Leicester, UK)
CHO M3 flip in phosphodef	Dr AB Tobin (University of Leicester, UK)
3T3-M1	Dr P Crespo (IIB, Santander, Spain)
Primary cultures	**Origin**
Rat adult cardiomyocytes	Obtained in the laboratory
Mouse neonatal cardiomyocytes	Obtained in the laboratory
Mouse neonatal cardiac fibroblasts	Obtained in the laboratory
Rat neonatal cardiomyocytes	Obtained in the laboratory
Rat neonatal cardiac fibroblasts	Obtained in the laboratory

2. METHODS

a. DNA manipulations

DNA cloning

Venus-YFP expression constructs for the protein complementation assay (PCA) were obtained as follows: GNAQ (mouse, accession number NM_002072) and PRKCZ (rat, accession number NM_022507.1) were subcloned into the 5´ and 3´-end of the Venus YFP PCA fragments, referred to here as N-terminal fragment (1 – 158 aa; F[1]) and the C-terminal fragment (159 – 239 aa; F[2]), respectively, as previously described (Remy & Michnick, 2004). Original cDNAs from pCDNA3.1(+)-HAPKCζ and pCIS-Gαq (see plasmid section) were PCR amplified with specific primers to insert compatible restriction sites at both ends of the PCR fragment. Due to the existence of internal restriction sites within PKCζ or Gαq of some of the enzymes to be utilised, those were replaced with alternative enzymes that generate compatible restriction sites. Both N- and C-terminal tagging options were generated for our proteins of interest to check for optimal expression and proper folding of the tagged protein. For cloning at the 5´end of the venus YFP fragment (*Not*I-*Cla*I sites), *PspOM*I-*Nar*I sites (compatible with *Not*I-*Cla*I sites) were inserted into 5´and 3´, respectively, of PKCζ and *Not*I-*Cla*I sites were inserted into 5´and 3´, respectively, of Gαq. Alternatively, for cloning at the 3´end of the venus YFP fragment (*Bspe*I-*Xba*I sites), *Bspe*I-*Xba*I sites were inserted into 5´and 3´, respectively, of PKCζ and *Bspe*I-*Nhe*I sites were inserted into 5´and 3´, respectively, of Gαq.

Renilla luciferase expression constructs for utilising in the protein complementation assay (PCA) were obtained as follows: GNAQ (mouse, accession number NM_002072) and PRKCZ (rat, accession number NM_022507.1) were subcloned into the 5` end of the 10-aa linker $(GGGGS)_2$ and of the Rluc-PCA fragments [Rluc-F(1):1–110aa; Rluc-F(2): 111–310aa of the humanised Rluc; pcDNA3.1] as described above.

DNA mutagenesis

PKCζ binding-deficient mutants, Gαq binding-deficient mutants and Gαq constitutively active mutants were prepared using the QuickChange site-directed mutagenesis kit (Stratagene). Complementary oligonucleotide pairs (usually 40-45 bp long) were designed to contain the desired point mutations in the middle of the primer sequence and had annealing temperatures higher than 78ºC and a minimum GC content of 40%. The primers were extended by PCR using the reagents provided in the kit under the following conditions: a total volume of 50 µl with 30 ng of template plasmid DNA, 1x reaction buffer, 100 nM of each primer, 1 µl dNTP mix and 2.5 U of *Pfu*UltraTM DNA polymerase was subjected to 16 cycles of a) 50 seconds denaturing at 95°C, b) 50 seconds annealing at 60°C and c) 6-8 minutes template elongation at 72°C. The PCR reactions were further incubated with 10 U DpnI restriction enzyme for 1 hour at 37°C to digest the parental DNA and directly transformed into 50 µl of *E. coli* XL1-Blue supercompetent cells (provided in the kit) and plated onto LB-ampicillin (50 µg/ml) agar plates. Resulting colonies were grown in LB broth-ampicillin (10 µg/ml) for plasmid preparation (Qiaprep Spin Miniprep Kit, Qiagen). Plasmids were subjected to DNA sequencing to verify the mutation and the fidelity of the whole cDNA sequence.

DNA agarose gel electrophoresis

Size determination and separation of DNA was achieved by agarose gel electrophoresis. 1% (w/v) of electrophoresis grade agarose (Invitrogen) was added to a volume of TAE buffer and heated in the microwave until molten. 0.6 µg/ml of ethidium bromide was then added and poured into the gel casting tray with comb. DNA samples were loaded into the wells in DNA loading buffer and run along-side of 1 µl of HindIII digestion fragments of λ and ø29 phages as DNA size markers. Gels were run at 0.01 Volts/mm^2 and DNA was visualised using a UV transilluminator.

Quantification of DNA

DNA was quantified by reading the absorbance of purified DNA at 260 nm using the DNA/RNA program of the BioRad Smart Spec™ spectrophotometer. DNA was appropriately diluted in dH_20 and a minimum of 200 µl placed in a quartz cuvette (Starna) with a path

length of 1 cm. Alternatively, the concentration of DNA samples was measured with a Nanodrop spectrophotometer and software (Thermo Scientific).

DNA digestion and 5' dephosphorylation

Approximately 1 µg of DNA was digested with 2 U of restriction enzyme for 1-3 hours in the enzyme buffer recommended by the supplier´s catalogue. Reactions were carried out in a 20µl volume and 0.2 mg/ml BSA was also added when appropriate. Linearised DNA vector was 5' dephosphorylated using 1 U of Alkaline Phosphatase (Invitrogen) in 1x dephosphorylation buffer. Dephosphorylation reactions were carried out in a final volume of 20µl at room temperature for 15 minutes and deactivated at 65 °C for 15 minutes.

DNA purification from agarose gels

DNA bands were excised from agarose gels using a sterile scalpel blade and DNA was extracted with the Quiagen® gel extraction kit following manufacturer´s instructions.

Ligation of DNA

Vector and insert DNA, both after digestion, were ligated together in a molar ratio of 1:3/1:6 using T4 ligase (Invitrogen) and the accompanying 5x buffer. Ligation was performed at room temperature overnight in order to maximise the number of ligations after which 2 µl of the ligation mixture were transformed into 50 µl of competent *E. coli* DH5-α cells and selected with the appropriate antibiotic.

Transformation of electrocompetent bacteria

50 µl of electrocompetent *E. coli* (XL1-Blue supercompetent cells, Stratagene, or electrocompetent DH5α cells) were thawed on ice for 10 min. Subsequently, 10 ng of plasmid were mixed with the bacteria and incubated on ice for 30 minutes. The bacterial cells were heat-shocked at 42 ºC for 45 seconds and returned to ice for another 2 minutes. 500 µl of pre-heated LB medium was added to the bacteria and then they were incubated at 37ºC for 1 hour with shaking (250rpm) to develop antibiotic resistance. The bacteria were spread onto agar plate containing the appropriate antibiotic and grown overnight at 37ºC in an inverted position.

PCR colony check

Colonies grown overnight were picked with a 20µl tip and resuspended in a PCR mix that contained a total volume of 50 µl with 1x reaction buffer, 100 nM of each primer, 1mM total dNTPs and 2.5 U of Taq (Biolabs) DNA polymerase. The remaining bacteria were streaked onto an LB-Ampicillin plate to obtain duplicates and incubated overnight

at 37ºC in an inverted position. The PCR was 1) 95ºC for 5 min, 2) 95ºC for 30 sec, 3) 55ºC for 30 sec, and 4) 72ºC for (1 min/Kb), followed by cycling from step 2 to Step 4 for 30 cycles, and by a final extension at 72ºC for 10 min. Resulting amplification products were run alongside the control vector in an agarose gel to check for the presence of the insert.

DNA sequencing

Isolated plasmids were sequenced by an external sequencing facility: Parque Científico (Madrid) or IRIC Genomic Platform (Montreal). Specific primers were utilised for Gαq and PKCζ was sequenced from the t7 promoter. The chromatograms were analysed with the Chromas Lite 2.1 software (http://technelysium.com.au/?page_id=13) and fasta files were analysed with Ape software (http://biologylabs.utah.edu/jorgensen/wayned/ape/) to annotate the final sequence.

Small-scale purification of plasmid DNA (Mini-Preps)

Small scale purification of plasmid DNA was carried out with the Wizard® Plus SV Minipreps DNA Purification System Protocol, following manufacturer´s instructions. Usually plasmids were resuspended in 30µl of dH_20 to reach a final concentration of 200ng/µl.

Large-scale purification of plasmid DNA (Maxi Preps)

Large-scale purification of plasmid DNA was performed using Endofree Plasmid purification kit® (Quiagen) according to the manufacturer's instructions. A saturated 250 ml culture of bacteria containing the plasmid of interest was grown overnight with aeration at 37ºC. Plasmid DNA was eluted into 5 ml of dH_20 and concentrated using ethanol precipitation before resuspension in 100 µl of dH_20. Typical DNA yield was 1 µg/µl.

Storage of bacteria

The long-term storage of bacteria containing transformed plasmids was at -80ºC in 10% glycerol.

DNA sequence alignment

Alignments of DNA sequences were performed using the Multiple sequence alignment (Multalin) software (Corpet, 1988) that can be accessed on http://multalin.toulouse.inra.fr/multalin/.

b. Culture of mammalian cell lines

Maintaining and subculturing the cells

Commonly used cell lines (specified above) were maintained in medium containing DMEM supplemented with 10% heat inactivated fetal calf serum (FBS, Invitrogen) glutamine 2mM and penicillin/streptomycin (0.01%/0.063%) at 37°C and 5% CO_2. CHO cells stably expressing muscarinic M1 and M3 receptors were grown in alphaMEM (Invitrogen, no nucleosides) with 10% FBS and 500µg/ml G418 for selection. In order to detach cells from the plate for subculturing, they were washed with PBS and incubated with trypsin for 5 minutes (0.5 ml of trypsin for 10 cm dish). Afterwards, cells were resuspended in 10ml of fresh media. The appropriate passage of cells was performed, usually ranging between 1:5 and 1:15, depending on the case.

Freezing/thawing

Cells from a confluent 10 cm dish were collected and resuspended in 1 ml of ice-cold freezing medium (90% fetal calf serum, 10% DMSO) and transferred to a 1.5 ml cryo-tube (Nunc). Cells were first stored at -80ºC for 2-3 days and then were transferred to liquid N_2 for long term storage. Cells were quickly thawed by warming them in a 37ºC waterbath. Next, the suspension was transferred into a 15 ml plastic tube (Falcon), 9 ml of prewarmed growth medium was added and the suspension was centrifuged at 1200 rpm for 5 minutes. The medium was aspirated to remove the traces of DMSO and the cells were resuspended in 10 ml of growth medium and plated in a dish.

Transient transfection

For transient transfections, cells were plated in 6 well, 6 cm, 10 cm or 15 cm dishes. The next day, cells were transfected with Lipofectamine/Plus reagents (Invitrogen). First, equal DNA amounts of different plasmid combinations were pipetted into 1.5ml tubes and resuspended in one volume of Optimem medium (Sigma) with Plus reagent. After 10 min of incubation, another volume of Optimem medium was mixed with the appropriate amount of Lipofectamine reaction and added to the DNA mix. Meanwhile, medium was removed from the plates and replaced with a minimal volume of Optimem. After 10 min of incubation, the solution was added to the plates. After 3 hours the transfection medium was removed to lower the cytotoxic effect of lipofectamine.

siRNA transfection

The siRNAs for PKCζ (mouse PRKCZ, NM_008860, On-target PLUS SMARTpool L-040823-00-0005, sequences GAUCGACCAGUCCGAAUUU, CAAGGCCUCACACACGUCUUA,

CAUCAAGUCUCAUGCCUUC, and GGGCAUGCCUUGUCCUGGA), clathrin (mouse CLTC, heavy chain), GAPDH (mouse) and scrambled used as a controls were obtained from Dharmacon (Lafayette, CO). NIH-3T3-M1 cells and cardiomyocytes were grown to 75% confluency and transfected with Dharmafect Reagent 1 (Dharmacon) according to the manufacturer's protocol.

Cell sorting with cytometer

When experiments required a homogeneous population of cells expressing the desired plasmid combination they were cotransfected with GFP and subjected to sorting with a cell cytometer. CHO-M3 cells were plated on 150cm^2 plates (Falcon) to reach a confluency of 80% for transfection after 24h. Different plasmids were cotransfected along with the pEGFP plasmid. In order to guarantee that all cells expressing GFP were also expressing the protein of interest the ratio between transfected μg of plasmid of interest/ pEGFP plasmid was kept to 8:1. Twenty-four hours after transfection, cells were detached from the plate with trypsin, resuspended in PBS and centrifuged. Cells were resuspended in sorting buffer (PBS, 5mM EDTA and 25mM HEPES pH 7 supplemented with 2% FBS) to reach a final concentration of 5 million cells per millilitre, and separated in the cytometer.

Collecting the cells

To collect the cells for different experiments they were washed with PBS and usually frozen at -20ºC for a short period of time until they were lysed. Alternatively, they were trypsinized with 0.5 ml of trypsin for 10 cm dish or 0.25 ml for 6 cm dish for 5 minutes and resuspended in 10 ml of fresh media. The suspension was centrifuged in a tabletop centrifuge at 1200 rpm for 3 minutes. The supernatant was discarded, and the cell pellet was resuspended in 10 ml of PBS to remove excess of PBS. Cells were centrifuged again for 3 minutes, the supernatant was discarded and the pellet was frozen.

c. Culture of mammalian primary cells

Primary cultures of neonatal mouse cardiomyocytes and fibroblasts were prepared from C57BL/6J mice or PKC-deficient mice or wild-type animals (SV129J background) by dissociation of neonatal (1–2 days) hearts. After excised, hearts were kept in Ca^{2+}-free HANKS medium at 4ºC and were mechanically disaggregated using a sterile scalpel blade. For the chemical disaggregation the tissue was transferred to a culture flask (T25) and kept in trypsin (0,05%) and type II collagenase (Worthington) for 15min at 37ºC after which the supernatant was collected and mixed with HANKS medium with calcium to stop the reaction. The macroscopical tissue fragments that remained in the flask were further disaggregated in up to 5 trypsinization steps. Each medium collected after these steps was

centrifuged (5min, 1500 rpm.), resuspended in 10% FBS-DMEM and kept at 37ºC. Upon conclusion of the disaggregation, cells were plated onto a conventional tissue culture plate (10cm) to allow cardiac fibroblasts to attach for 2 h. After, cardiomyocyte-containing supernatant was plated in M12 multiwell plates precoated with 0.02% gelatin (300,000 cells per well) and cultured in DMEM plus 10% serum for 48 h. Cardiomyocytes were cultured for 2 days before use, whereas cardiac fibroblasts were expanded and passaged twice before the assay to remove contamination by endothelial cells. Both cell types were cultured in DMEM (D5648, Sigma- Aldrich) supplemented with 10% FBS.

Isolation of adult cardiac myocytes was performed as previously described (Fernández-Velasco et al., 2003). Adult male C57BL6 mice or Wistar rats were heparinized (4 units/g) and anesthetized with sodium pentobarbital (50 mg/kg). The hearts were removed and mounted on a Langerdorff perfusion apparatus. The ascending aorta was cannulated, and a retrograde perfusion was set up. The hearts were perfused successively with the following oxygenated solutions at 36 °C: 1) standard nominally Ca^{2+} Tyrode solution (3min) and 2) standard nominally Ca^{2+}-free Tyrode solution (15 min) containing type II collagenase (Worthington). The hearts were removed from the Langerdorff apparatus, and after removal of atria, the ventricles were cut off and gently shaken for 3 min in a standard Tyrode solution containing 0.1 mM $CaCl_2$ to disperse the isolated cells. The resulting cell suspensions were filtered through a 250mm nylon mesh and centrifuged for 4 min at 20G. Finally, the cell pellets were stored in Tyrode solution containing 1mM $CaCl_2$.

d. Cell treatments

Primary neonatal cardiac cells were stimulated with angiotensin II (100 nM) in serum-free medium, during the indicated time periods. The cells were serum-starved for 5–6 h before ligand addition to minimize basal kinase activity. Treatment with the PKCζ pseudosubstrate inhibitor (10μM) was initiated 30 min before agonist stimulation. For the inactivation of Gi proteins, cells were pretreated with pertussis toxin (100 ng/ml) for 16 h.

Primary adult cardiac myocytes were stimulated in Tyrode solution containing 1mM $CaCl_2$ shortly after extraction.

NIH-3T3-M1 cells were stimulated with carbachol (10μM) after being serum-starved for 5-6h.

CHO cells stably expressing muscarinic receptors were stimulated with carbachol at different concentrations after being serum-starved for 2h.

e. Preparation of cell lysates

To prepare cellular lysates, collected cells were resuspended in 100-1000 µl of ice-cold lysis buffer with protease and phosphatase inhibitors (Roche), transferred to a precooled 1.5 ml reaction tube and incubated in a rocker (250rpm) for 1 hour. RIPA lysis buffer was utilised in immunoprecipitations with PKCζ, MEK5, Gαq and GRK2. For the detection of phosphorylation, a low detergent lysis buffer (ERK5 buffer) was preferred. Afterwards, the lysates were centrifuged for 10 minutes at 4ºC in a microfuge (14000 rpm). The cleared lysates were transferred to fresh precooled 1.5 ml reaction tubes and stored at -80ºC.

f. Determination of protein concentration

The protein concentration in cellular lysates was determined using the DC Protein Assay Reagents A, B and S (Bio-Rad). Three µl of cellular lysates were mixed with 25µl of solution obtained by the combination of 10 µl DC Protein Assay Reagents S and 500 µl of Reagent A. Afterwards, 200 µl of Reagent B was added and the solution was incubated 10 minutes at room temperature. Absorbance at 750 nm was measured in a Biorad iMark microplate Reader. Protein concentration was determined with respect to the absorbances of known concentrations of BSA protein. In general, 40-60 µg of total protein were used for western blotting.

g. Immunoprecipitation

For immunoprecipitations, 20µl of anti-HA agarose beads (Santa Cruz) or 0.5µl of GRK2 monoclonal antibody were incubated with 1 mg of cell lysates for 3 h at 4ºC. In the case of GRK2, protein A/G (Santa Cruz), was added and incubated for another hour. The beads were then washed 5-7 times in RIPA or ERK5 buffer and analyzed by immunoblotting.

h. Immunoblotting

Cellular lysates/immunoprecipitations were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) on a 6-12% Laemmli gel for cellular lysates. After electrophoresis, the proteins were transferred from the polyacrylamide gels to nitrocellulose membranes (0.2 µm, Whatman) using a wet blotting apparatus (Bio-Rad). The efficiency of transfer was always evaluated by staining with Ponceau S staining solution for 2 minutes. Ponceau S was washed out with TBS-tween and the membranes were blocked for 1 hour in TBS-tween supplemented with 5% BSA at room temperature, or alternatively, for detecting endogenous protein phosphorylation PhosphoBLOCKER™ Blocking Reagent (Cell Biolabs, Inc) was used. Membranes were incubated

with primary antibodies in TBS-Tween supplemented with 3% BSA overnight at 4ºC (see Materials for antibodies dilution). Afterwards, the blots were washed three times for 10 minutes in TBS-Tween followed by incubation with the secondary antibodies 1 hour at room temperature (see Materials for antibodies dilution). After extensive washing with TBS-Tween, secondary antibodies bound to the proteins on the membranes were detected using ECL (Enhanced ChemiLuminiscence) from Amersham Pharmacia Biotech and Agfa films. Alternatively, in the case of fluorescent secondary antibodies, the Odyssey Infrared Imaging System was utilsed. Bands were quantified using by laser densitometry with a Biorad GS-700 scanner.

i. GST pull-down

GST-pull down cell lysates were incubated with 20µl of Glutathione Sepharose 4B resin (GE Healthcare life sciences) overnight. Washing procedure and analysis was similar to immunoprecipitations.

j. Immunohistochemistry and Histological Analysis of PKCζ KO and wild-type mice

The generation of PKC knock-out mice (SV129J background) has been described previously (Leitges et al., 2001). Littermate wild-type and PKCζ-/- male mice (32 weeks of age) were subjected to continuous infusion of angiotensin II (or PBS as a control) for 14 days, a well established model for the induction of cardiac hypertrophy. Angiotensin II dissolved in PBS was continuously and subcutaneously infused at a rate of 432g/kg/day using Alzet osmotic mini-pumps (model 2002, Alza Corp., Mountain View, CA) implanted dorsally under isofluorane anesthesia Several physiological parameters were measured before sacrifice but are not detailed here as they are part of a different PhD thesis (Carlota García-Hoz, 2008). Hearts from wild- type or knock-out mice were excised and cleaned of blood to be fixed in formalin and embedded in paraffin wax. Sections of 5µm were processed for immunohistochemistry.

To compare the expression of Ets-1 and the activation of MEF2C and MEK5 in wild-type versus knock-out mice, a high temperature antigen unmasking technique (10-min microwaving of slides in Tris-EDTA, pH 8.0, for 90 s) was carried out. Antigen retrieval was performed after deparaffinization to enhance staining. Sections were then incubated with 5% horse serum for 30 min, and then washed three times with sterile PBS (pH 7.5) prior to incubation with the appropriate primary antibodies at 1:50/1:100 dilutions. Biotin-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and used at 1:4000 dilution. For all antibodies, signal was amplified using avidin peroxidase (ABC Elite Kit Vector) and visualized using diaminobenzidine as a substrate

(DAB kit, Vector Laborato- ries). Finally, sections were stained with hematoxylin. Image analysis was performed with ImageJ 1.46a software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Four images were acquired from randomly selected locations in each slide. Random RGB images were converted to 8-bit images for the quantification of the DAB signal to obtain the integrated density parameters (Wang et al., 2010).

k. Inositol phosphate assay

Twenty-four hours after transfection with the desired combinations of cDNA constructs, HEK-293 cells were seeded in 24-well plates at a density of 4×10^5 cells per well. Cells were labelled overnight with 5 μCi/ml myo-inositol (Perkin Elmer) in inositol-free 10% FBS DMEM. Labelled cells were washed with pre-warmed PBS, detached with trypsin and centrifuged in a microfuge tube. Then, cells were resuspended in inositol-free DMEM plus 10 mM LiCl and incubated at 37 °C for 15 min. The reactions were stopped by centrifugation, removal of the incubation buffer and addition of ice-cold 6% perchloric acid/1.5mM EDTA. Phenol red pH indicator was added to the sample to reach a final concentration of 0.5μg/ml and the pH was adjusted to 7.0 with 10 M KOH. The cell debris was removed by centrifugation (5 min at room temperature in a microcentrifuge). The supernatants, containing the intracellular inositol phosphates, were applied to AG1-X8 ion-exchange chromatography columns (200–400 mesh, formate form). Each column was washed with two volumes of unlabeled inositol (5 mM) and two volumes of 5 mM sodium tetraborate/60 mM ammonium formate. The total inositol phosphates fraction was eluted with 3 ml of 0.1 M formic acid/1.5 M ammonium formate and quantified by liquid scintillation counting, as previously described6. Incorporation of radioactive titrated inositol phosphate by cell populations expressing different constitutively active mutants was normalised to the cpm counts of control cells (pCDNA3 transfected).

l. Protein Complementation Assay (PCA)

In the protein-fragment-complementation assay (PCA), protein-protein interactions (PPIs) are measured by fusing each of the proteins of interest (A and B) to complementary N- or C- terminal regions of a reporter protein. Upon transfection of the constructs and expression in mammalian cells, if the proteins A and B interact, the reporter fragments are brought together, fold into the native structure of the reporter and reconstitute its activity. PCA reporter proteins have been chosen as those producing a variety of detectable activities, including fluorescent, luminescent, and colorimetric signals, as well as simple survival selection assays (Remy & Michnick, 2007). Common features for this assay include the ability to detect protein-protein interactions *in vivo* and *in vitro* in any cell type, in appropriate subcellular compartments or organelles, and it allows detection

of interactions that are specifically induced in response to developmental, nutritional, environmental, or hormone-induced signals.

Fluorometric analysis and fluorescence microscopy of Venus-PCA

Equal amounts of CHO-M3 cells grown in 6-well plates were co-transfected with the Venus YFP PCA expression vectors (pcDNA3.1) coding for prey-F[1] and / or bait-F[2] using lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, cells were subjected to fluorimetric analysis and fluorescence microscopy. For the fluorimetric analysis cells were washed with PBS, gently trypsinized and resuspended in 200 µl of PBS. Total cell suspensions from each well were transferred to 96-well black microtitre plates (Dynex; VWR Scientific,Mississauga, Ontario) and subjected to fluorometric analysis (integration time 10sec, excitation wavelength 470nm, emission wavelength 528nm) (Spectra MAX GEMINI XS; Molecular Devices, Sunnyvale, CA). The background fluorescence intensity corresponding to non-transfected cells was subtracted from the fluorescence intensities of all of the samples. For fluorescence microscopy experiments cells were grown in low-fluorescent media (HBSS, Invitrogen) and images were taken using a Nikon Eclipse TE2000U inverted microscope with 40x objective and YFP filter cube (41028, Chroma Technologies). Images were captured with a CoolSnap CCD camera (Photometrics) using Metamorph software (Molecular Devices). In the case of continuous monitoring of fluorescence upon the addition of a ligand, cells were kept in a closed chamber at 37ºC.

Bioluminescence measurement in Renilla luciferase PCA

Twenty-four hours after transfection, cells grown in 6-well plates were starved for 2 hours with serum-free DMEM. After, they were detached and resuspended in 500µl of PBS. A total of 100 µl of each cell suspension (aprox 10^5 cells) was transferred (in triplicates) to 96-well white-walled plates (Corning) and transferred to a preheated (37ºC) LMaxII384 luminometer (Molecular Devices). Carbachol was added to the wells and endpoint bioluminescence measurements were made at different times by addition of the Renilla luciferase substrate, benzyl-coelenterazine (5µM, Nanolight). Rluc activity was monitored for the first 10 seconds after addition of the substrate (integration time 10sec, post-injection delay 5 sec, peak emission wavelength 482 nm).

m. Real-time monitoring of cell adhesion, spreading, proliferation and cell viability using the xCELLigence System.

Cells were seeded in 96-well gold electrode sensor plate (E-plates) pre-coated with fibronectin (10 mM) and monitored using the xCELLigence system RTCA SP instrument (Roche Applied Science). The conceptual basis of this technology is that cells, when attached to the plate, act as mini-condensers and change the electrical impedance that is being continuously recorded by means of several electrodes located underneath the plate. Cellular impedance was converted to a cell index (CI) that allows for the assessment of different cellular processes. As cells attach and proliferate over time, impedance rises as does the cell index recorded. Changes in their adherence, viability and morphology also change the impedance and can thus be monitored. Therefore, cell proliferation, cell surface coverage, cellular adhesion strength or cell viability can be measured in a real-time label-free cellular setting.

This technique has been previously shown to strongly correlate with classical methods for the detection of these cellular processes. The effect of the cytotoxic drug paclitaxel was measured by the XCELLigence system and the Sulforhodamine B (SRB) assay showing a good correlation (Limame et al., 2012). Similarly, cell migration was assayed by Matrigel-coated Transwells and crystal violet staining, which strongly correlated with data obtained through the XCELLigence system. The measurement of cell viability is usually performed by tetrazolium salt-based colorimetric assays (MTT, XTT, WST-1) that account for the number of active mitochondria in a cell population. Additionally, apoptosis can be measured by DNA fragmentation assays. It was recently demonstrated that the effect of two cytotoxic drugs (5-FU and MG-132) with different cell-killing kinetics was reliably monitored with the XCELLigence system compared to classical detection methods (Ke et al., 2011). Thus, this novel detection system allows for continuous label-free monitoring of cellular processes, allowing for the selection of the optimal timepoints for the performance of end-point classical assays.

For each experiment growth media baseline impedance was measured in each well before the addition of the cells to ensure all changes recorded were normalised to those initial values determined by the ionic composition of the media. Approximately 4000 cells were added per well and monitoring started shortly afterwards. Changes in cell index were measured each 15min for varying times (70-150hrs).

In order to define the specific parameters to be measured in each experiment a careful analysis of the global kinetics was performed. The first 16h after the cells were plated were excluded from every analysis as they correspond to the adhesion phase. Usually within 6h the cells were already attached, however, 16h was chosen in order to be confident

about the complete competence of the cells in response to treatments. No differences in adhesion were observed between the different populations. The specific analysis for carbachol and cell viability experiments is explained below.

Measurement of carbachol-induced effects

Carbachol at varying concentrations was usually added 16h after plating the cells. Two different parameters were measured; cell blebbing and cell growth arrest. Cell blebbing was analysed for 1h after carbachol addition when invariably all the populations reached a maximum increase in the cell index. This effect is transient and within 2-3h all the populations had decreased the cell index to basal levels before carbachol addition. The parameter chosen to express the blebbing capacity of each cell population was expressed as follows:

$$\text{Slope [blebbing]} = \frac{\text{Ci [max]- Ci [intial]}}{\Delta\text{time}}$$

Where Ci [max] is the maximum cell index, Ci [initial] is the cell index before the addition of carbachol and Δtime is the time difference between Ci [initial] and Ci [max].

Cell growth arrest promoted by carbachol was always measured a minimum of 6h after carbachol addition to exclude blebbing from the proliferation calculation. Proliferation was measured in all populations for 24h and was expressed as follows:

$$\text{Slope [growth]} = \frac{\text{Ci [24h]- Ci [0h]}}{\Delta\text{time}}$$

Where Ci [24h] is the cell index after 24 hours, Ci [0h] is the cell index at the time-point when proliferation measure starts (usually 6h after the addition of carbachol) and Δtime is the time difference between Ci [initial] and Ci [max].

The negative effect of carbachol on cell growth was detected for a maximum of 72h after which time cells invariably started to proliferate again.

Measurement of long-term cell viability

All cell populations were monitored for varying times (70-150hrs) until reaching a maximum cell index (inflexion point) after which it rapidly decreases, which is indicative of cell death. In no case cell death was due to excessive confluency as confirmed by plate inspection with a microscope and by the different cell indexes at which cell death was initiated in each experiment. Apoptosis entry time was expressed as the time between the start of the experiment and the inflexion point.

n. Statistical analysis

In all cases, experiments were analysed for statistical significance by a two-tailed T test. Asterisks are indicative of the following significances: * = $p<0.05$, ** = $p<0.005$, *** = $p<0.001$.

o. Bioinformatics modelling

Structural models were generated with Pymol software at the Bioinformatics Unit (CBMSO). Chain Q (corresponding to Gαq) of the crystal structure of the Gαq-GRK2-Gβγ complex (PDB accession number: 2BCJ) was utilised.

IV. RESULTS

1. THE Gαq/PKCζ/ERK5 AXIS IN THE CARDIOVASCULAR SYSTEM

At the start of this thesis, the biochemical mechanism of the activation of ERK5 by GPCRs had recently been unveiled in our laboratory. The identification of PKCζ as a novel Gαq effector provided the link between Gq-coupled GPCRs and the ERK5 pathway. It was extensively demonstrated that the presence and activity of PKCζ was essential for the activation of the ERK5 pathway by Gq-coupled receptor ligands such as carbachol and sphingosine-1-phosphate in epithelial cells (see section 4d in the Introduction). The process did not involve the classical Gαq effector PLCβ, nor cytoplasmic tyrosine kinases or EGF receptor transactivation. Since Gαq is a crucial protein in cardiovascular function, we sought to determine the occurrence of this signalling axis in the heart.

a. Angiotensin-mediated activation of ERK5 in cardiomyocytes requires PKCζ

We sought to determine whether a key messenger acting through Gq-coupled GPCR such as angiotensin II also triggered the ERK5 cascade in different cardiac cell types. Primary cultures of neonatal mouse cardiomyocytes were incubated with angiotensin II for different periods of time and endogenous ERK5 activation was assessed by immunoblot analysis using an ERK5 antibody that detects ERK5 stimulation by the appearance of a band of slower electrophoretic mobility, corresponding to the phosphorylated, stimulated kinase (Fig. R1A). A clear, time-dependent increase in endogenous ERK5 activation was detected in response to this agonist. Our previous results in epithelial cells indicated that a functional association between Gαq and PKCζ was essential for stimulation of the ERK5 pathway by Gq-coupled GPCR agonists. To establish whether this novel pathway was also operating in cardiomyocytes, primary neonatal cell cultures were treated either with

scrambled or PKCζ-targeting siRNA oligos. Interestingly, whereas a clear ERK5 activation (assessed using a specific phospho-ERK5 antibody) was noted in cardiomyocytes treated with scrambled siRNA, angiotensin-mediated endogenous ERK5 stimulation was not detected upon siRNA-induced PKCζ downregulation (Fig. R1B). We also investigated ERK5 stimulation by angiotensin in cardiomyocytes isolated from PKCζ-deficient mice or wild-type littermates (SV129J background). As in cardiomyocytes obtained from the C57BL/6J strain, angiotensin promoted a clear ERK5 activation in cells from wild-type, with an earlier peak of activation, whereas this response was absent in PKCζ-/- cardiomyocytes (Fig. R1C).

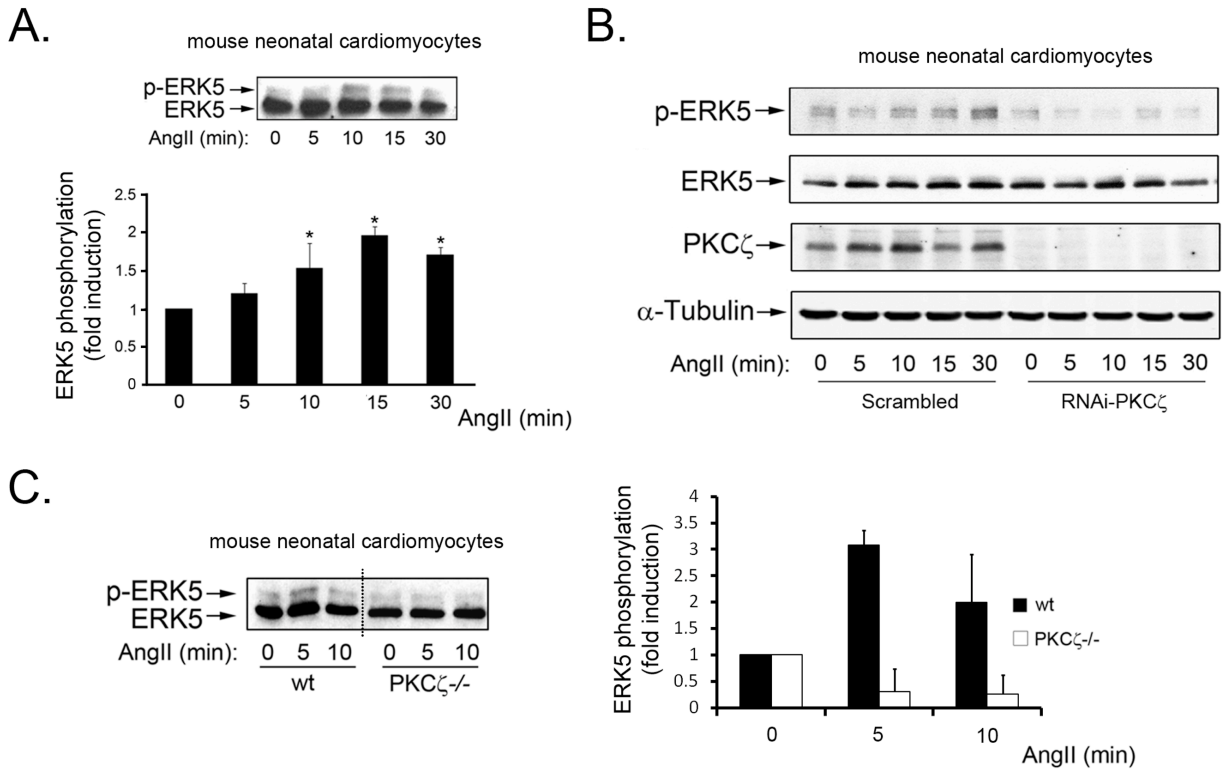

Figure R1. Stimulation of Gq-coupled GPCR by angiotensin II promotes ERK5 activation in neonatal cardiomyocytes in a PKCζ-dependent manner. (A) Neonatal mouse cardiomyocytes primary cultures were incubated with 100 nM Angiotensin II for the indicated times, and endogenous ERK5 activation determined with an ERK5-antibody that recognises both the phosphorylated (upper band) and un-phosphorylated (lower band) forms of ERK5 as detailed in Methods. Data (mean ± SEM of 3 independent experiments) were expressed as fold-activation compared to the absence of agonist (*$p<0.05$, two tailed T-test). (B),(C), Angiotensin II-induced ERK5 activation is abrogated upon PKCζ down-regulation in neonatal mouse cardiomyocytes. (B), Primary cardiomyocytes were treated with scrambled or PKCζ-targeting siRNA oligos as detailed in the Methods section, and then challenged with angiotensin 100 nM for the indicated times. ERK5 activation was determined in cell lysates with a phospho-ERK5 specific antibody. Along with PKCζ expression, total ERK5 and alpha-tubulin levels were also determined as loading controls. (C), Primary cultures of cardiomyocytes were obtained from wild-type (wt) or PKCζ-deficient (PKCζ-/-) mice as detailed in the Methods section, and challenged with angiotensin 100 nM for different time periods. ERK5 activation was monitored and quantified and data (mean ± SEM of 3 independent experiments, with activation in the absence of angiotensin taken as control condition) expressed as percentage of the phosphorylated kinase (p-ERK5) versus total ERK5.

It is well established that cardiac myocytes undergo dramatic phenotypical and functional changes from birth until becoming fully developed cells (Ahuja et al., 2007) (Fig. R2A). Hence, studies in neonatal cardiac myocytes may not reflect the situation in the adult mice. Thus, we sought to determine whether the Gαq-ERK5 pathway was conserved in adult cardiomyocytes. We found that angiotensin-dependent ERK5 stimulation could also be observed in cardiomyocytes isolated from adult mouse (Fig. R2B) or rat (Fig. R2C) and that this process was blocked in the presence of the specific PKCζ inhibitor (the cell-permeable myristoylated PKCζ pseudosubstrate peptide) (Fig. R2C). Collectively, data in figures R1&2 indicate that PKCζ is strictly required for Gαq-mediated ERK5 stimulation in cardiomyocytes.

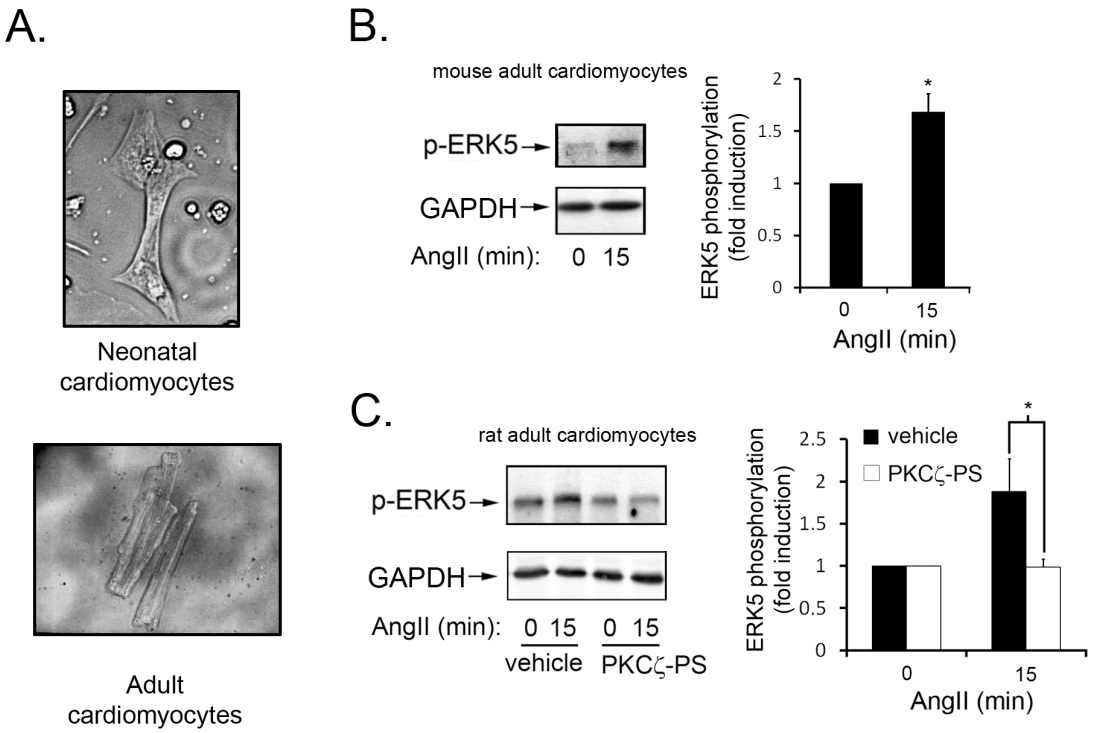

Figure R2. The AngII-initiated Gαq-PKCζ-ERK5 pathway is operative in adult cardiac myocytes. Primary cultures of adult mouse (A) or rat (B) cardiomyocytes were isolated as detailed in the Methods section and challenged with Angiotensin 100 nM for 15 min. (B), Adult rat cardiomyocytes were preincubated with vehicle or a specific PKCζ pseudosubstrate inhibitor (10 μM). ERK5 activation was detected in cell lysates with a phospho-ERK5 specific antibody, and data was normalised using GAPDH as loading control and expressed as fold-induction over basal conditions. Data are mean ± SEM of 3 independent experiments (*p<0.05, two tailed T-test).

b. Angiotensin-mediated activation of ERK5 in cardiac fibroblasts requires PKCζ

Although cardiac myocytes make up the bulk of the myocardial volume, cardiac fibroblasts are the most numerous cell type in the heart and play essential roles in myocardial function. Thus, we also sought to ascertain whether angiotensin could promote ERK5 activation in cardiac fibroblasts in a similar fashion as in cardiomyocytes. Indeed, acute angiotensin II treatment promoted a clear stimulatory time-course of endogenous ERK5 in cardiac fibroblasts (Fig. R3A). In line with the data in cardiomyocytes, the pretreatment of primary neonatal cardiac fibroblasts prior to agonist challenge with a PKCζ inhibitor markedly decreased endogenous ERK5 phosphorylation in response to angiotensin II (Fig. R3B). Also, since paracrine transmodulation of Gi-coupled-GPCR has been described to operate in certain pathways of cardiac fibroblasts, we treated the cells with pertussis toxin (PTX) to inhibit Gi-mediated signalling. In this context, ERK5 activation by angiotensin II in cardiac fibroblasts was not affected (Fig. R3C). Together, these data suggest that PKCζ is also required for Gαq-mediated ERK5 activation in cardiac fibroblasts.

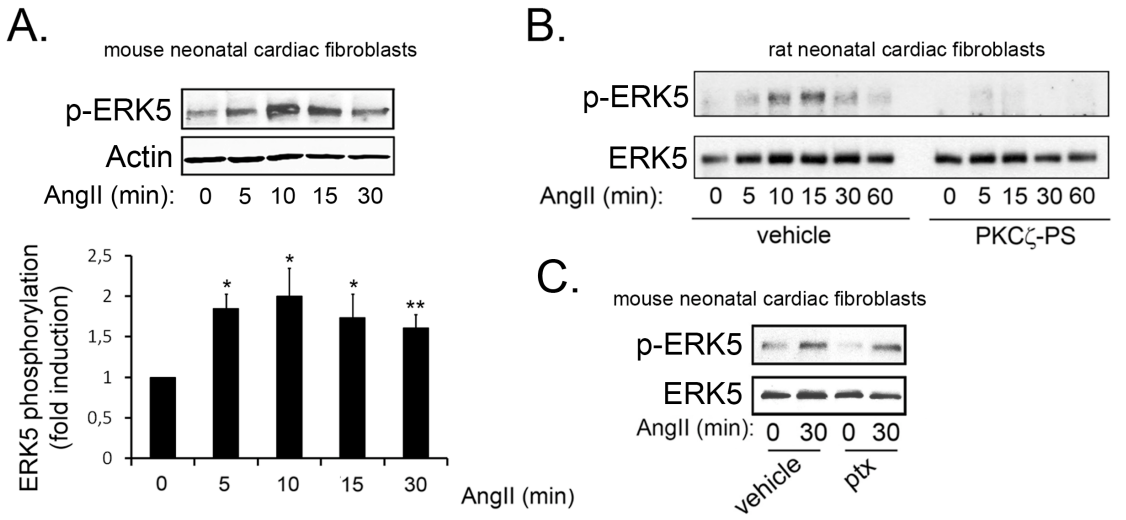

Figure R3. The AngII-initiated Gαq-PKCζ-ERK5 pathway is operative in neonatal cardiac fibroblasts. (A) Neonatal mouse cardiac fibroblasts were challenged with angiotensin (AngII), and ERK5 stimulation was determined in cell lysates with a phospho-ERK5 specific antibody. Data (mean +/- SEM of 3 independent experiments) were normalised using actin as a loading control and expressed as fold induction compared with the absence of agonist (*p<0.05, **p<0.005, two tailed T-test). Rat (B) or mouse (C) cardiac fibroblasts were preincubated with vehicle or with a myristoylated PKCζ pseudosubstrate (PS) inhibitor (10μM, B) or pertussis toxin (Ptx 100ng/ml, C) and challenged with 100 nM angiotensin for the indicated times. ERK5 activation was determined as in A. Blots are representative of 2-3 experiments.

c. The angiotensin Gq-coupled receptor activates ERK5 through PKCζ *in vivo*

To assess the potential relevance of the Gαq/PKCζ pathway in cardiac cells *in vivo*, we subjected homozygous null mice (PKCζ-/-) and matched control littermates (wild-type) to chronic angiotensin II (or control vehicle) infusion for 14 days using osmotic mini-pumps. This is a classical, well-established procedure to assess angiotensin effects in cardiac functions and to promote cardiac hypertrophy in mice models (Ikeda et al., 2005). At the end of the infusion period we performed an immuno-histological analysis of the hearts using specific antibodies against known upstream and downstream elements of the ERK5 cascade (Fig R4A). The use of phosphospecific antibodies showed that MEK5 (the upstream activator of ERK5) and MEF2C (a well-established downstream target of ERK5) display a higher phosphorylation state upon angiotensin treatment in wild-type animals that is absent in PKCζ-deficient mice (Fig. R4B). Similarly, the expression of Ets-1 (a known target of MEF2c) was augmented in wild-type animals as opposed to PKCζ KO mice (Fig. R4B). Quantification of the DAB staining (see methods) confirmed the significant difference in the ERK5 pathway activation markers upon angiotensin treatment between wild type and PKCζ-/- animals (Fig. R4C).These data further indicate that PKCζ is an important mediator of ERK5 activation by Gq-coupled GPCRs in murine heart *in vivo* and support that the Gαq/PKCζ/ERK5 pathway plays a relevant role in the response triggered by angiotensin II.

As part of a different PhD thesis in our laboratory, the physiological response of these animals to angiotensin was studied and revealed that the Gαq/PKCζ/ERK5 signalling axis had an essential role in the development of cardiac hypertrophy programmes in response to this challenge (see introduction, section 5d) (García-Hoz et al., 2010).

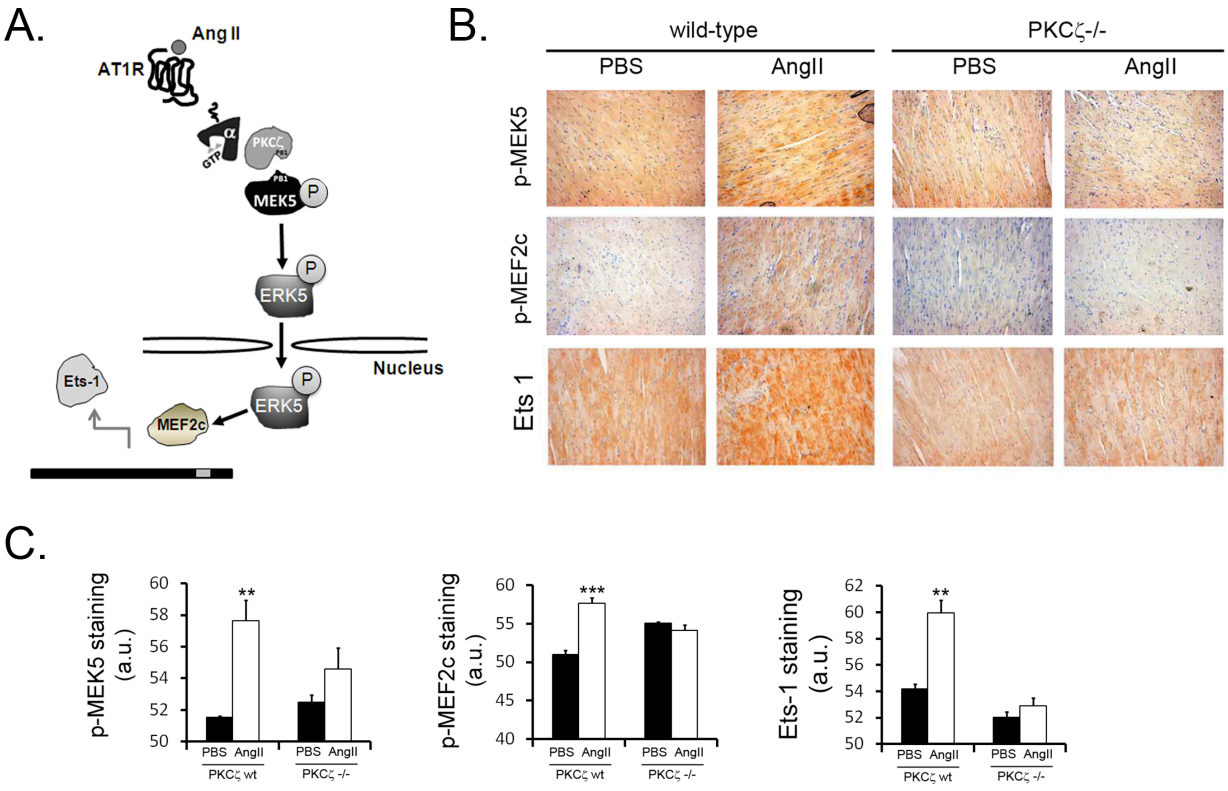

Figure R4. Angiotensin II triggers the Gαq-PKCζ-ERK5 pathway *in vivo* (A) Cartoon depicting the steps of the AngII-ERK5 pathway in the heart leading to the activation of MEF2c and to the transcription of Ets-1. (B) PKCζ -/- and matched wt control littermates were subjected to chronic angiotensin II (AngII) infusion (or PBS, as a control vehicle) for 14 days using osmotic minipumps as detailed in Methods. Sections of hearts from each mouse population were analysed through immunohistochemistry. The activation status of the pathway was assessed through MEK5 and MEF2C stimulation by phosphorylation and expression levels of Ets-1. Brown shading indicates positive DAB staining. Signal intensity was quantified as detailed in the Methods section and data plotted below represent the average of 3 independent experiments (**p<0.005; ***p<0.001, two tailed T-test). a.u., arbitrary units.

2. CONTRIBUTION OF Gαq/ β-ARRESTIN SIGNALLING ROUTES TO THE ACTIVATION OF ERK5 BY GPCR

Once established the relevance of the pathway in the heart, we aimed to identify the specific contribution of Gαq and β-arrestin signalling branches downstream of GPCRs towards the activation of ERK5. Since β-arrestin has been recently proposed as an alternative signalling node to heterotrimeric G proteins, it is essential to ascertain whether a known GPCR-initiated pathway like ERK5 is equally or differentially activated by these two signalling players. This is especially relevant in the heart where alternative Gαq- or β-arrestin-initiated pathways can have opposite physiological effects (see introduction, section 1d).

a. ERK5 activation by Gq-coupled GPCR does not require receptor internalisation

In order to facilitate the study of the mechanistic basis of GPCR-ERK5 activation we initially utilised different cell lines overexpressing Gq-coupled muscarinic receptors. This has been widely proven to be the most successful experimental approach to study ERK5 phosphorylation due to the poor quality of currently available detection methods. In particular, the NIH-3T3 cell line that stably overexpresses the M1 muscarinic receptor (NIH-3T3-M1) has been extensively used to detect endogenous ERK5 activation.

Upon activation, GPCRs internalise in a process that will lead to recycling or degradation of the receptor (see introduction, section 1d). Recently, these receptor desensitisation processes have been reported to also provide alternative ways for signalling propagation via β-arrestins. Since β-arrestin recruitment leads to GPCR internalisation in a process that virtually always involves the participation of clathrin, we determined the activation of the ERK5 pathway in a clathrin knock-down scenario. Thus, NIH-3T3-M1 cells were pretreated with scrambled siRNA or clathrin-targeting oligonucleotides and stimulated with the muscarinic agonist carbachol for 30 minutes (Fig. R5). Results indicated that ERK5 phosphorylation was unaffected in the absence of clathrin thus suggesting that clathrin-mediated receptor internalisation was not required for this process.

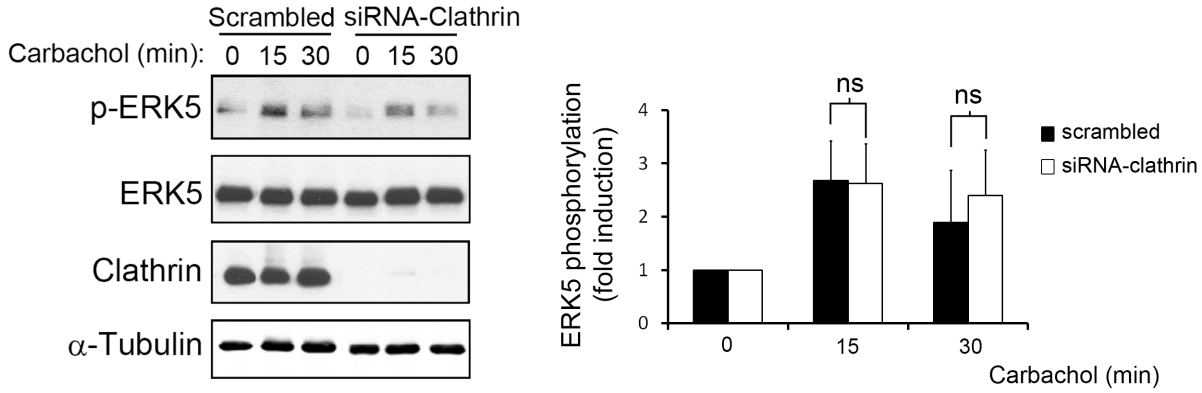

Figure R5. Carbachol-induced activation of the Gαq-PKCζ-ERK5 pathway does not require clathrin-dependent internalisation processes. NIH-3T3 cells stably overexpressing the muscarinic M1 receptor (NIH-3T3-M1 cells) were transfected with siRNA oligonucleotides targeting clathrin heavy chain or non-targeting scrambled oligonucleotides as detailed in the Methods section. After 48h of incubation, cells were serum-starved for 5h and challenged with carbachol (10μM) for the indicated times. ERK5 phosphorylation was assessed in cell lysates with a phospho-ERK5 specific antibody. Data (mean +/- SEM of 3 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction over basal conditions. Clathrin and alpha-tubulin expression levels were also determined (ns: non significant).

Since other alternative internalisation processes have been reported for GPCRs, we sought to determine whether they played a role in ERK5 activation. To that purpose, we utilised a Cho cell line that overexpresses an internalisation-deficient mutant M3 muscarinic receptor. This receptor harbours several mutations on the third internal loop of the

receptor (motif SASS to AAAA) that prevent trafficking into endosomes (Torrecilla et al., 2007). It is noteworthy that both M1 and M3 muscarinic receptors couple to Gαq with very similar affinity and both have recurrently been used as models to study Gαq signalling. In the Cho cell lines used throughout this thesis, ERK5 is invariably transfected and immunoprecipitated due to the scarcity of ERK5 molecules in this cell type and the additional difficulty posed by the poor quality of phosphoantibodies available for this protein. Thus, we transfected HA-tagged ERK5 into Cho-M3 and Cho-M3-internalisation-deficient cells and stimulated with carbachol at different times. Surprisingly, we detected an increased and sustained activation of ERK5 by the internalisation deficient mutant compared to control (Fig. R6). This further confirmed that ERK5 activation by muscarinic receptors occurs independently of receptor internalisation.

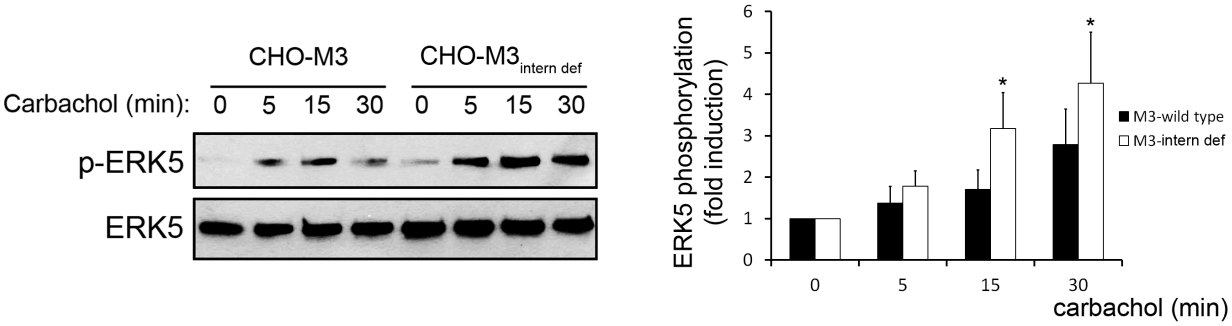

Figure R6. Carbachol-induced activation of the Gαq-PKCζ-ERK5 does not require receptor internalisation. Cho cells stably overexpressing wild-type muscarinic M3 receptor or internalisation-deficient M3 receptor (characterised in (Torrecilla et al., 2007)) were transfected with ERK5-HA. Twenty-four hours after transfection, cells were serum-starved for 2h and stimulated with carbachol (10μM). ERK5-HA was immunoprecipitated with an anti-HA agarose-conjugated antibody as detailed in the Methods section. ERK5 phosphorylation was assessed in the immunoprecipitate using a phosphospecific antibody. Data (mean +/- SEM of 3 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction over basal conditions (*p<0.05, two-tailed T-test) .

b. ERK5 activation by Gq-coupled GPCRs does not involve receptor phosphorylation or β-arrestin recruitment

β-arrestin is known to be recruited to activated GPCRs through a mechanism dependent on GRK2-mediated phosphorylation of the receptor. Thus, the translocation of arrestins to the plasma membrane occurs through the recognition of phosphorylation sites on the internal loops of receptors. To this point, we had determined that receptor internalisation was not required for ERK5 activation which consistently suggested that, if β-arrestin participates in ERK5 activation, this is not occurring in the endosomes. However, it remained the possibility that β-arrestin could initiate pathways leading to ERK5 activation from the plasma membrane. In order to ascertain this, we utilised a Cho cell line overex-

pressing a phospho-deficient muscarinic M3 mutant receptor. This receptor, in which all the phosphorylatable serines within the internal loops are mutated to alanine, has been described to be completely unable to recruit βarrestins but to be otherwise functional (Kong et al., 2010). Notably, acetylcholine-induced ERK5 activation by the phosphodeficient M3 receptor was augmented compared to wild-type M3 receptor as shown in Fig. R7A. To further confirm this, we used a similar phosphodeficient version of the M1 receptor and stimulated with submaximal doses of acetylcholine in order to highlight the differences in ERK5 activation by wild-type or mutant receptor. Indeed, we observed a clear enhancement of ERK5 activation by the phosphodeficient mutant M1 receptor (Fig. R7B). Taken together, these data suggest that the phosphorylation of the receptor and the subsequent recruitment of β-arrestins are not involved in ERK5 activation by Gq-coupled GPCRs. On the contrary, downregulation of these processes seems to favour the activation of the ERK5 pathway.

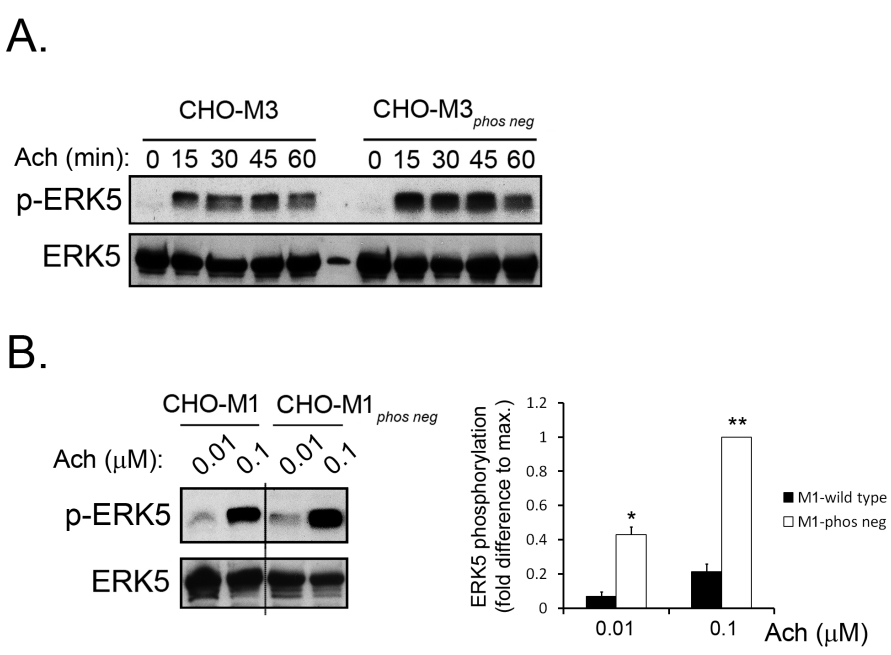

Figure R7. Acetylcholine-induced activation of the Gαq-PKCζ-ERK5 does not require receptor phosphorylation. Different stable lines of Cho cells (previously characterised in (Kong et al., 2010)) that overexpress wild-type or phosphorylation-deficient muscarinic M3 receptor (A) and wild-type or phosphorylation-deficient muscarinic M1 receptor (B) were utilised. All cell lines were transfected with ERK5-HA. Twenty-four hours after transfection, cells were serum-starved for 2h and stimulated with acetylcholine (100μM) for the indicated times (A) or incubated for 15min with acetylcholine at various concentrations (B). ERK5 phosphorylation was assessed as in Fig. R6. In (A) representative blot of two independent experiments is shown. In (B), data (mean +/- SD of 2 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction over maximum activation (*p<0.05, **p<0.005; two-tailed T-test).

c. ERK5 activation by GPCRs is Gαq-biased

The data above indicated that Gαq-initiated signalling is the key participant in ERK5 activation by muscarinic Gq-coupled GPCR. We sought to confirm this notion by studying the activation of ERK5 in a Gαq-enriched cell population. In these circumstances,

Gαq-dependent pathways would be potentiated whereas β-arrestin-dependent pathways would either remain unaltered or be downregulated as a result of a functional competition. Interestingly, the activation of ERK5 in Cho-M3 cells overexpressing Gαq was greatly enhanced compared to control (Fig. R8). This confirms that Gαq activation is the predominant event in the ERK5 pathway.

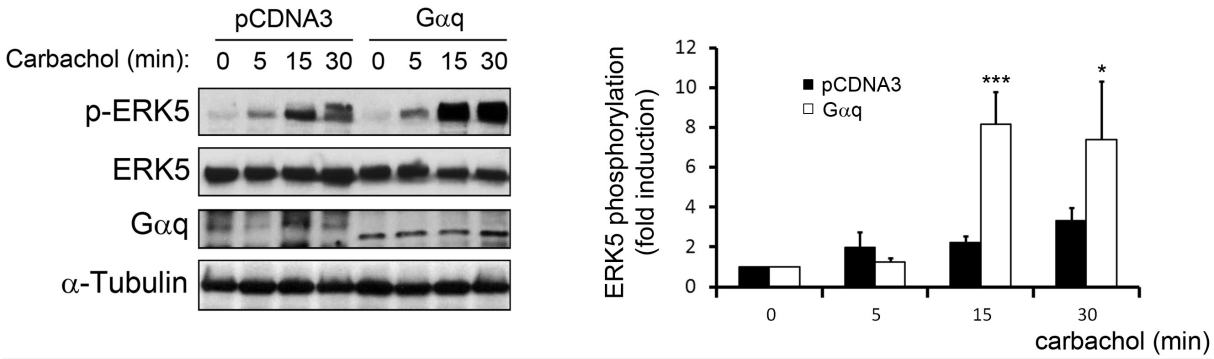

Figure R8. Gαq overexpression enhances ERK5 activation by carbachol. Cho cells stably expressing wild-type muscarinic M3 receptor (Cho-M3 cells) were transfected with ERK5-HA and either pcDNA3 or Gαq. Twenty-four hours after transfection, cells were serum-starved for 2h and stimulated with carbachol (10μM) for the indicated times. ERK5 phosphorylation was assessed as in Fig. R6. Data (mean +/- SEM of 3 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction over basal conditions (*p<0.05, **p<0.005, Two-tailed T-test).

To further establish the biased nature of this pathway we utilised the angiotensin receptor ligand SII (Sar1-Ile4-Ile8 (SII) Ang II), which displays biased properties towards βarrestin-pathways. As shown in Fig. R9, the ERK5 pathway was not activated by the SII ligand compared to the unbiased natural ligand angiotensin II in primary cardiac fibroblasts. Collectively, data in fig R5-R9 suggest that ERK5 activation by muscarinic and angiotensin receptors is Gαq-biased.

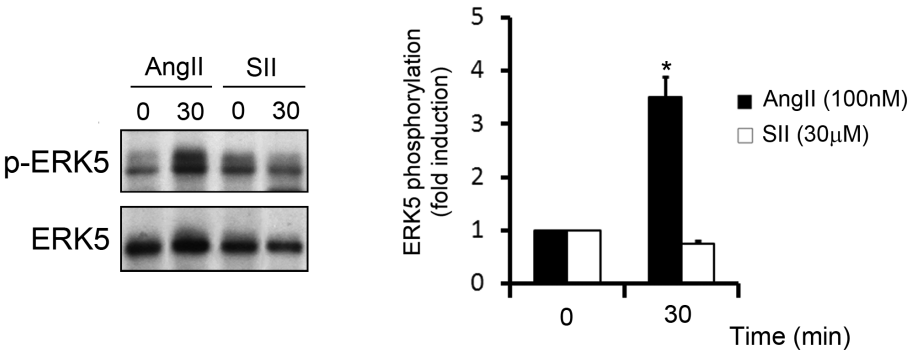

Figure R9. ERK5 is not activated by a β-arrestin-biased angiotensin receptor agonist in cardiac fibroblasts. Neonatal mouse cardiac fibroblasts were challenged with angiotensin (AngII, 100nM) or Sar1-Ile4-Ile8-Ang II (SII, 30mM) for 30 minutes, and endogenous ERK5 stimulation was determined in cell lysates with a phospho-ERK5 specific antibody. Data (mean +/- SEM of 3 independent experiments) were normalised using total ERK5 as a loading control and expressed as fold induction compared with the absence of agonist (*p<0.05, two tailed T-test).

3. THE Gαq/PKCζ COMPLEX IN LIVING CELLS

Previous data suggested that the initial and key event for the activation of the ERK5 pathway by Gq-GPCRs is the formation of a complex between Gαq and the novel effector PKCζ (García-Hoz et al., 2010). In order to further dissect the mechanisms of the pathway, we aimed at the detailed characterization of this protein-protein complex.

a. The Gαq/PKCζ complex is specifically detected in living cells

We had previously shown that a Gαq/PKCζ complex was transiently formed upon agonist stimulation (as indicated by immunoprecipitation experiments) and that these purified proteins could interact directly (García-Hoz et al., 2010). Thus, we sought to complement the *in vitro* characterization with an approach that allowed detecting the interaction in living cells. To that purpose we implemented the protein complementation assay (PCA) with the fluorescent protein venus-YFP, an engineered and more fluorescent version of YFP. The assay consisted in expressing PKCζ and Gαq tagged to cognate fragments of YFP (N- or C-terminal halves) in living cells. Interaction events between PKCζ and Gαq would bring the YFP fragments together thus allowing reconstitution of the native fold of the fluorescent protein (see scheme in Fig. R10A). Therefore, fluorescence is a direct measure of the number of protein-protein complexes formed and, additionally, the irreversible nature of the assay allows easy detection and quantification of otherwise transient complexes.

A known high-affinity interaction (Zipper dimerisation) was used as a positive control in the venus-YFP PCA assay. Furthermore, we also analysed the potential formation of complexes between Gαq and another member of the PKC family (PLCβ) and also between PKCζ and another member of the Gα family (Gαi1), to assess the specificity of the interaction. As shown in Fig R10b and R10c, the Gαq/PKCζ complex can be readily detected in living cells compared to control and it is specific since neither PLCβ nor Gαi are able to reconstitute the fluorescence (e.g. to interact) with Gαq and PKCζ, respectively. This provides the *in vivo* confirmation to similar results from coimmunoprecipitation experiments (García-Hoz et al., 2010)

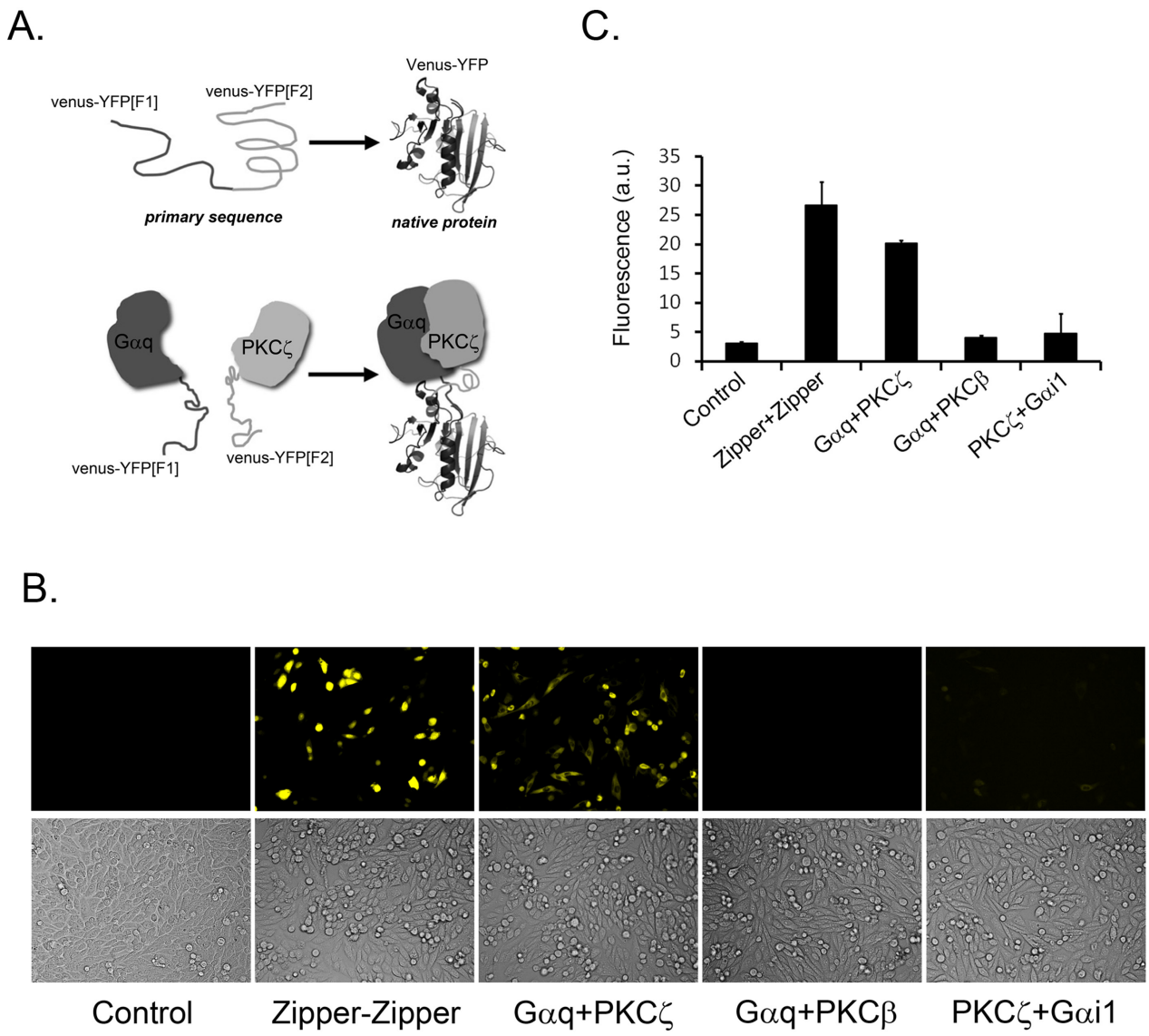

Figure R10. The Gαq/PKCζ complex is specifically detected in living cells. (A) Conceptual explanation of the venus-YFP-based protein complementation assay (PCA). This technique utilises the reconstitution of a fluorescent protein from N/C-terminal halves that are fused to two proteins of interest. Fluorescence upon expression of the protein pair in living cells is thus a measure of the occurrence of an interaction between the proteins, which brings cognate fragments of YFP close enough to allow reconstitution of the native fold. The irreversible nature of fluorescent protein YFP-PCA assays allows for easy trapping and visualisation of transient complexes. (B) The interaction between Gαq and PKCζ occurs in living cells and is family-specific since PLCβ, as a control for other PKCs, and Gαi, as a control for other Galpha subunits, do not interact with Gαq and PKCζ, respectively. Cho-M3 cells were transfected with different pairs of protein complementation assay (PCA) plasmids that express proteins fused to cognate fragments of venus-YFP: Control (PKCζ-Venus YFP[F1]+pcDNA3), Zipper+Zipper (Zipper-Venus YFP[F1]+ Zipper Venus YFP[F2]), Gαq+PKCζ (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]), Gαq+PLCβ (Gαq-Venus YFP[F1]+ PLCβ-Venus YFP[F2]), PKCζ+Gαi (Gαi-Venus YFP[F1]+ PKCζ-Venus YFP[F2]). Twenty-four hours after transfection cells were visualised under the microscope as detailed in the Methods section. Representative brightfield and YFP fluorescence images at 40x magnification are shown. (C) Fluorimetric analysis of the populations above was performed as detailed in the Methods section and data (mean +/- SEM of 3 independent experiments) were normalised with respect to control.

b. PKCζ does not associate with Gα11, the other ubiquitous member of the Gαq/11 family.

The Gαq/11 family is composed of four members of whom the most notable and ubiquitous are Gαq and Gα11. To date, Gαq and Gα11 are thought to play very similar roles and to act on the same cellular effectors. Thus, by performing a PCA-based approach we initially aimed to confirm that the novel effector PKCζ would indifferently associate with Gαq and Gα11. Surprisingly, whereas PKCζ readily associated with Gαq, it did not achieve a significant fluorescent signal when coexpressed with Gα11 as demonstrated by microscopy images and fluorescence quantification (Fig. R11A). In order to confirm the lack of association detected in living cells we performed an immunoprecipitation approach. Similarly, when HA-tagged PKCζ is immunoprecipitated Gαq is clearly detected in the multimolecular complex, whereas this is not the case for Gα11 (Fig. R11B).

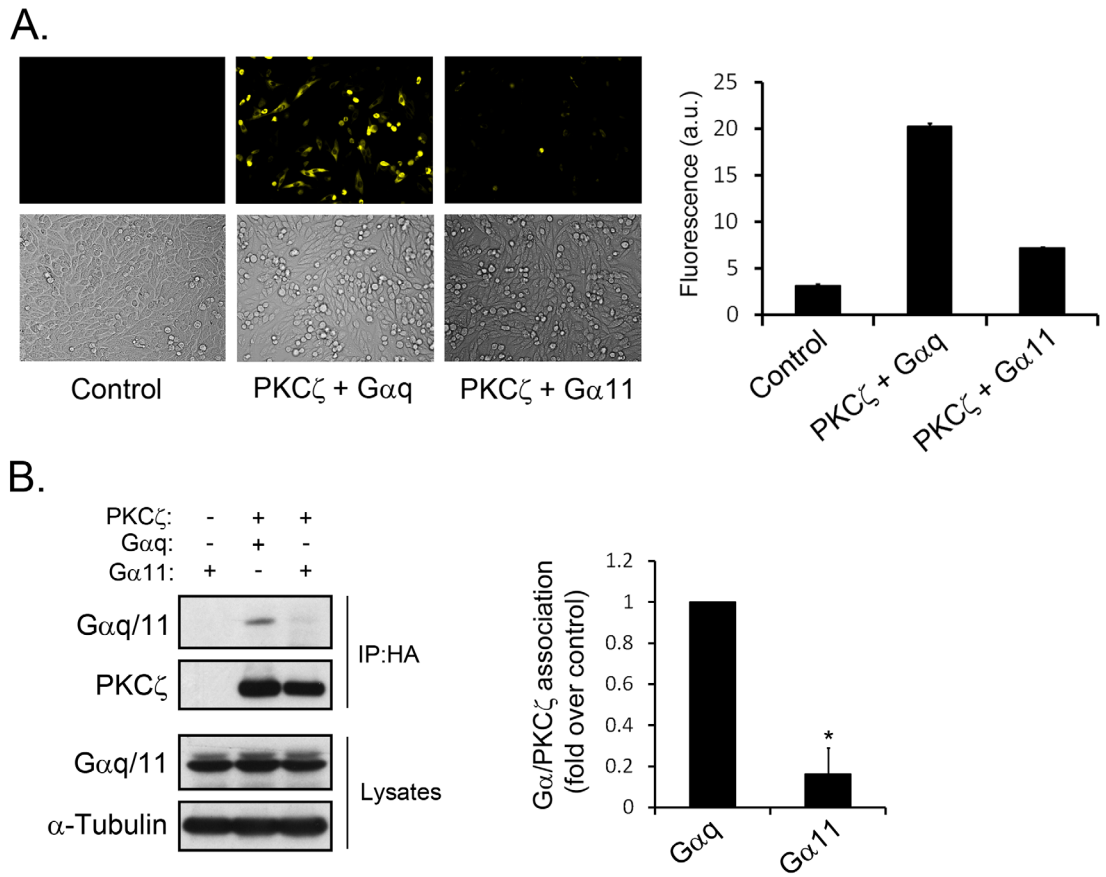

Figure R11. PKCζ selectively interacts with Gαq but not with Gα11. (A) Venus-YFP-based PCA assay was performed as in Figure R10 in Cho-M3 cells: Control (PKCζ-Venus YFP[F1]+pcDNA3), Gαq+PKCζ (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]), PKCζ+Gα11 (Gα11-Venus YFP[F1]+ PKCζ-Venus YFP[F2]). (B) COS-7 cells were transfected with different combinations of plasmids encoding HA-PKCζ, Gαq and Gα11. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. To compare the association of the different Gαq/11 family members to PKCζ, blot bands were quantified and normalised by total HA-PKCζ. Data (mean +/- SEM of 3 independent experiments) were normalised taking the Gαq/PKCζ wt association as a control.

Taken together, data in Figures R10 and R11 indicate that the Gαq/PKCζ complex is selectively detected in living cells and seems to be Gαq-specific since the closely related member of the Gαq/11 family, Gα11, is not able to interact with PKCζ. Indeed, PKCζ could be the first differential Gαq/Gα11 effector to be identified.

4. IDENTIFICATION OF THE INTERACTION SURFACES INVOLVED IN THE Gαq/PKCζ COMPLEX

We next aimed to characterise the interaction surfaces involved in Gαq/PKCζ association. The identification of such binding surfaces could provide an additional level of information with regards to the functionality and the biochemical mechanisms involved in the activation of this pathway.

a. The PB1 domain of PKCζ is essential for Gαq association and ERK5 activation

Initially, we characterised the interaction surface within PKCζ. This protein displays a three-dimensional structure (Fig. R12A) in which the catalytic site is obstructed by an autoinhibitory domain called pseudosubstrate (see Introduction, section 3b). Additionally, it harbours a C1 and a PB1 domain (Fig. R12A). PB1 domains are known protein-protein interaction domains and, in PKCζ, this module alone accounts for the majority of known interactions (Moscat & Diaz-Meco, 2000; Sumimoto et al., 2007). Hence, we sought to determine whether this domain was involved in the activation of ERK5 by Gq-coupled GPCRs. NIH-3T3-M1 cells were transfected with GST-tagged PKCζPB1 domain or GST alone as a control and the activation of ERK5 in response to carbachol was assessed (Fig. R12B). The overexpression of PKCζ-PB1 domain had a dominant-negative effect and was sufficient to abrogate ERK5 activation. Consistent with a key role of this PKCζ region, PB1 domain overexpression interfered with the formation of the Gαq/PKCζ complex (Fig. R12C), thus suggesting that PKCζ associates with Gαq through its PB1 domain.

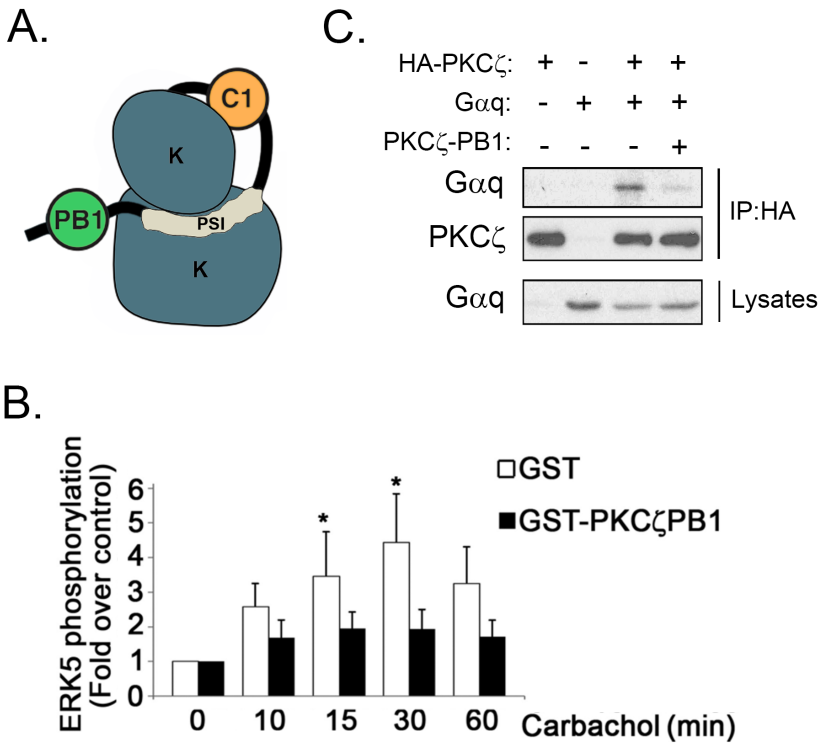

Figure R12. Overexpression of the PKCζ-PB1 domain abrogates GPCR-mediated ERK5 activation and Gαq/PKCζ interaction. (A) Cartoon depicting the three-dimensional structure of PKCζ showing the different domains: PB1, PSI (pseudo-substrate inhibitory region), C1, K (kinase domain). (B) NIH-3T3-M1 cells were transiently transfected with the PB1 domain of PKCζ fused to GST (aa1-122 of PKCζ). Forty eight hours after transfection, cells were challenged with 10 μM carbachol for different times. Endogenous ERK5 activation was determined with a phospho-specific antibody. Data (mean +/- SEM of three independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction over basal conditions (*p<0.05, two tailed T-test). (C) COS-7 cells were transfected with different combinations of plasmids encoding HA-PKCζ, Gαq and GST-PKCζPB1. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies to determine the coimmunoprecipitation of Gαq and PKCζ in the presence/absence of PKCζ-PB1 domain. A representative blot of 3 independent experiments is shown.

b. Lysine 19 is a crucial residue for PKCζ to interact with Gαq

The PB1 domain of PKCζ, as that in PKCλ or p62, is composed of a PB1-type I (acidic) and a PB1-type II (basic) domain. It is predicted that type I and II dimerise/oligomerise providing signalling platforms in several cellular processes (see Introduction, section 3b). We initially sought to determine whether the PB1-type II domain of PKCζ was driving the interaction with Gαq. A rationale-based mutagenesis strategy was designed (Fig. R13) to mutate aminoacids that had been determined crucial in other PB1-type II interactions in the literature (Hirano et al., 2005). Lysine in position 19 has been pointed as an invariable aminoacid in all PB1-PB1 structures (Hirano et al., 2004, 2005) and to co-ordinately work with other basic residues located around the lysine. Additionally, residues around position 90 in the primary sequence are known to locate nearby the lysine in the three-dimensional structure and contribute to form the binding surface (Fig. R13).

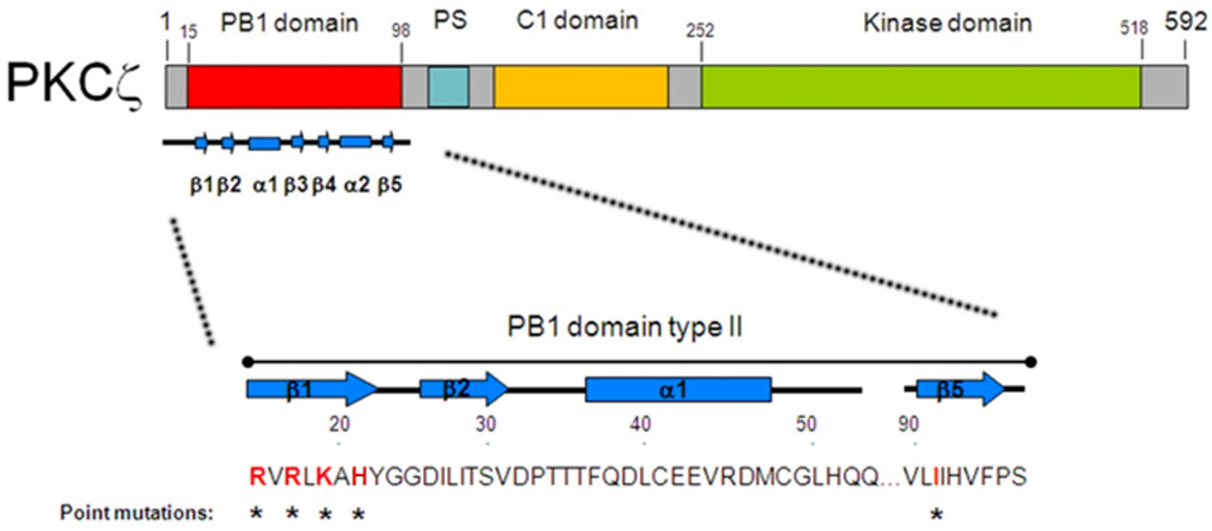

Figure R13. PKCζ-PB1 domain analysis and mutagenesis strategy. Cartoon depicting the primary sequence of PKCζ with different domains highlighted, and a specific focus on the PB1 type II domain. The PB1 type II domain, known to participate in ERK5 activation through an interaction with MEK5, was targeted for directed point mutagenesis. Selected residues are homologous to previously identified interaction-driving aminoacids in other PB1-type II harbouring proteins (Hirano et al., 2005).

Based on Fig. R13, several alanine point mutations were generated in the PB1-type II domain of PKCζ. Mutant PKCζ forms were overexpressed in COS-7 cells together with Gαq wild-type and immunoprecipitated with an anti HA antibody. The presence of co-precipitated Gαq with the different mutants was assessed by western-blot as shown in figures R14A&B. The most significant decrease in the interaction could be ascribed to the PKCζ-K19A mutation that alone accounted for a 70% decrease in the amount of coprecipitated Gαq (Fig. R14A). Other PB1-type II aminoacids such as R15 or I92 also seemed to participate in the interaction. Interestingly, the PKCζ-H21A mutant had an enhanced ability to associate to Gαq (Fig. R14B), which supports the involvement of that surface in the interaction. Additionally, it was demonstrated that lysine 19 in PKCζ is also essential for its interaction with MEK5 (Fig. R14C), as predicted by other PB1-PB1 structures. Taken together, these data indicate that the PB1 domain type II of PKCζ, and particularly lysine in position 19, is key for binding to both Gαq and MEK5.

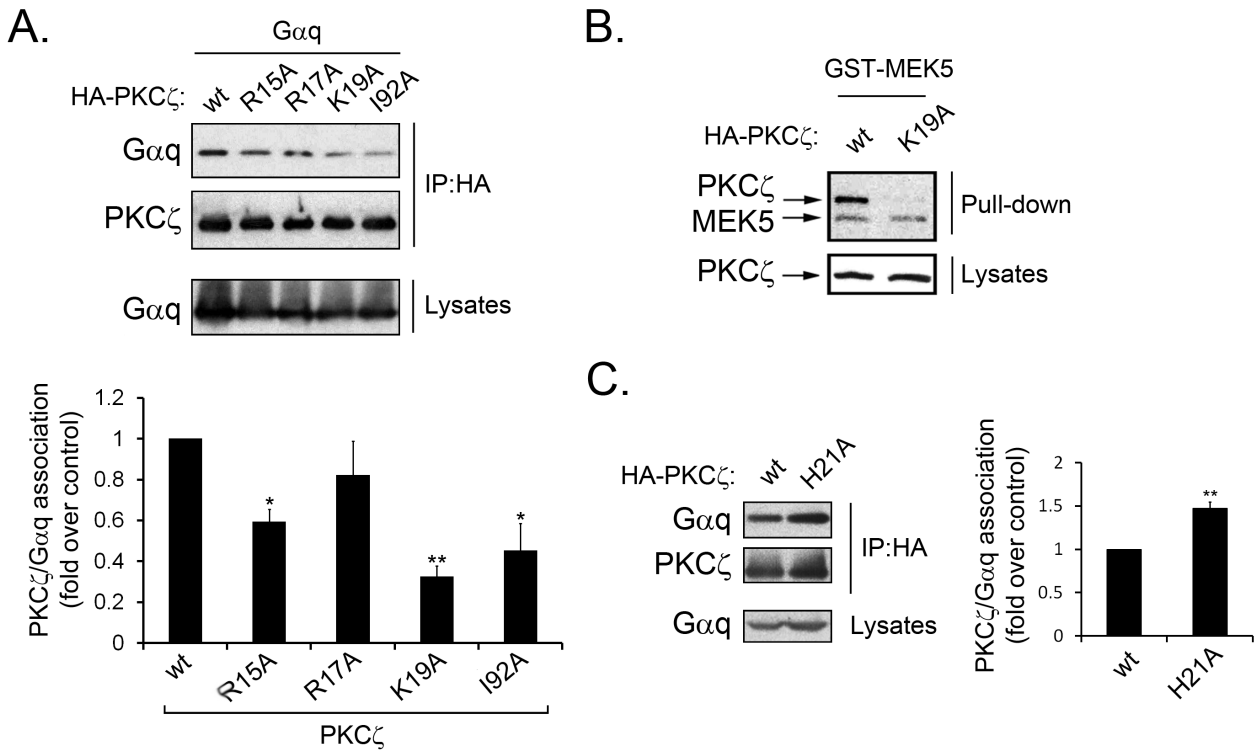

Figure R14. Identification of key residues in PKCζ required for the interaction with Gαq. Alanine-scanning mutagenesis was performed in PKCζ following the strategy depicted in Fig. R13. (A, C) COS-7 cells were transfected with combinations of plasmids encoding Gαq and different HA-PKCζ mutants. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. To compare the association of Gαq to the different PKCζ mutants, blot bands were quantified and normalised by total HA-PKCζ. Data (mean +/- SEM of three independent experiments) were normalised with respect to Gαq/PKCζ wt association as a control. (B) COS-7 cells were transfected with different combinations of plasmids encoding MEK5-GST, HA-PKCζ and HA-PKCζK19A and a pull-down with glutathione sepharose was performed to precipitate MEK5-GST as described in the Methods section. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies to determine the association of MEK5 to PKCζK19A mutant. Representative blot is shown of 3 independent experiments (*p<0.05, **p<0.005, two tailed T-test).

c. The C-terminus of Gαq is required for association to PKCζ

In order to start delineating the region of Gαq that is involved in PKCζ binding we utilised the previously generated information on the specificity of this complex. We have shown that members of the Gαi family cannot interact with PKCζ either in vitro (García-Hoz et al., 2010) or in living cells (Fig R10). Thus, we utilised two different chimeras in which the C-terminus (aa222-353) of either Gαq or Gαi1 had been substituted by that of Gαi1 and Gαq, respectively (Fig. R15A). We co-expressed the two different chimeras together with HA-PKCζ in COS-7 cells and immunoprecipitated with an anti-HA antibody (Fig. R15B). It was clearly shown that the substitution of Gαq C-terminus by that of Gαi1 completely abolished the association with PKCζ thus indicating that the interaction determinants are predominantly located in this region.

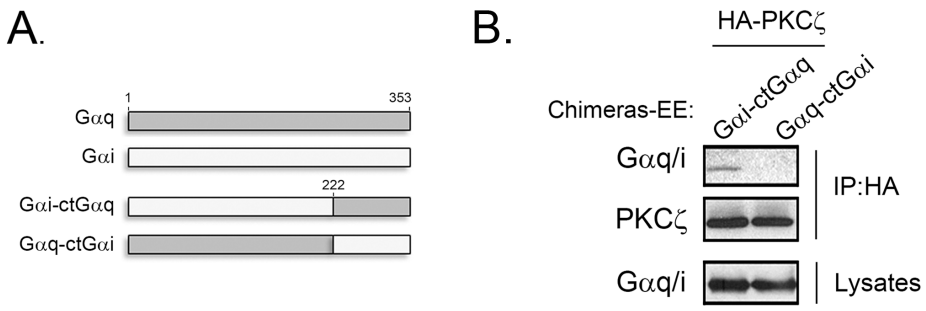

Figure R15. The C-terminus of Gαq (aa222-353) is essential to interact with PKCζ. Chimeric constructs of Gαq and Gαi (known to not interact with PKCζ) were utilised to delineate the region of Gαq that contains the determinant to interact with PKCζ (A) Cartoon depicting the arrangement of the Gαq/Gαi chimeras. (B) COS-7 cells were transfected with different combinations of plasmids encoding EE-tagged chimeras (Gαi-ctGαq, Gαq-ctGαi) and HA-PKCζ mutants. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific anti-HA and anti-EE antibodies to determine PKCζ association to the different chimeras. A representative blot of 3 independent experiments is shown.

d. Identification of a pseudo-PB1 domain in the Gαq effector-binding region

In order to carry out a rationale-based mutagenesis strategy, a careful analysis of binding surfaces was performed in the C-terminal region of Gαq. A highlight of common binding surfaces in Gαq is shown in Figure R16. Crystal structures and mutagenesis studies of Gαq have contributed to identifying the crucial aminoacids involved in known Gαq complexes (underlined in different colours in Fig. R16). In this regard, we performed several sequence alignments between known PKCζ-interacting partners and Gαq. The comparison between MEK5 PB1-type I domain and the effector binding region of Gαq showed some striking similarities, with predominance of acidic residues at the β4-α3 loop. Since we had previously established that PKCζ interacts with Gαq through its PB1-type II domain, we hypothesised that Gαq could harbour a pseudo-PB1 type I domain that would be responsible for the interaction. Additionally, PB1-PB1 interactions have been shown predominantly electrostatic between positively and negatively charged surfaces and it was tempting to suggest that a similar phenomenon would be occurring between lysine/arginine residues in PKCζ PB1-type II and glutamic/aspartic acids in the Gαq pseudo-PB1-type I domain.

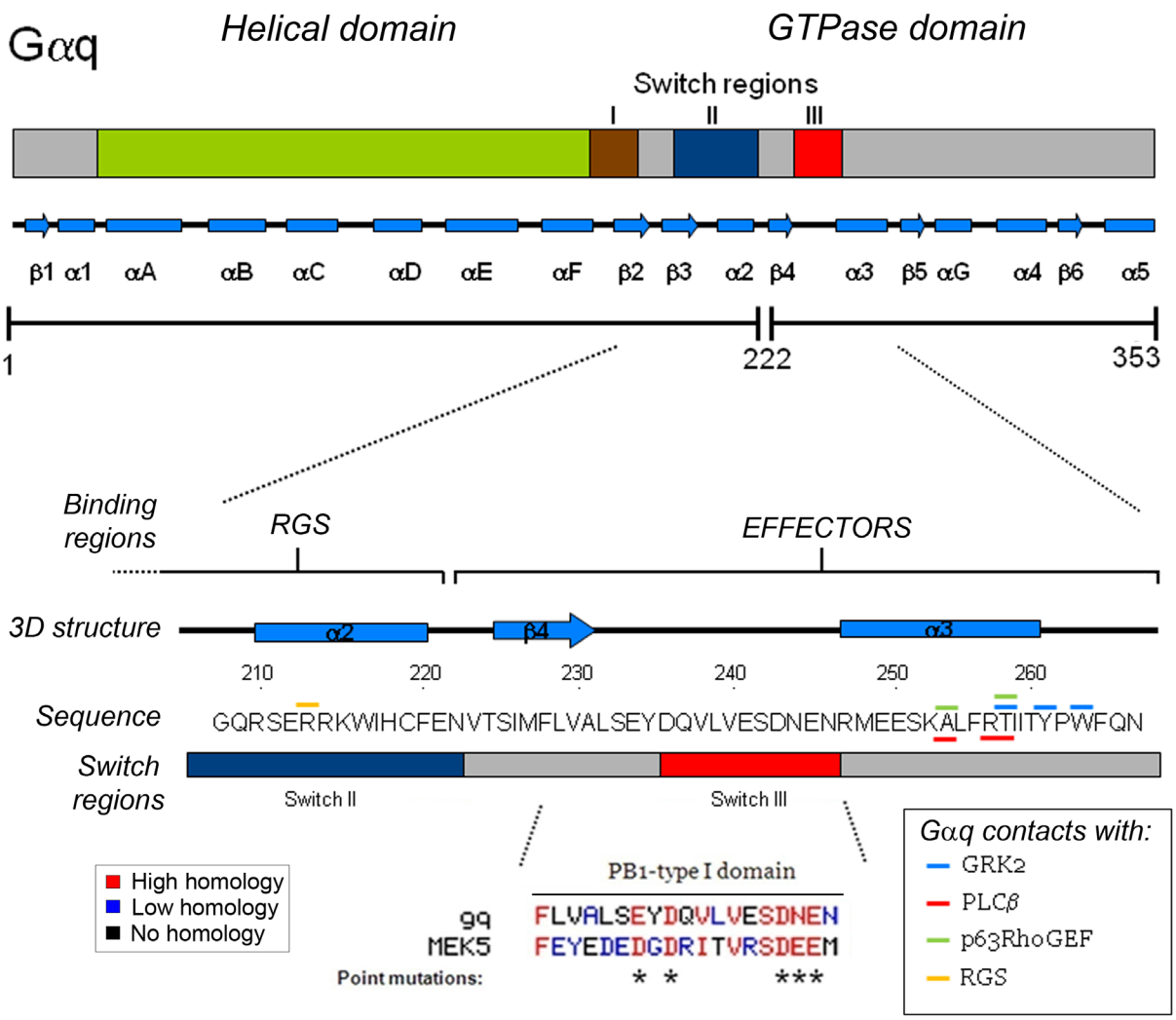

Figure R16. Identification of a pseudo-PB1 domain in the Gαq effector-binding region. Cartoon depicting the different domains of Gαq and the corresponding secondary structure motifs. From top to bottom: Full-length Gαq highlighting the helical and GTPase domains. The GTPase domain harbours the switch regions that undergo profound conformational changes upon GTP binding. The two regions (1-222, 222-353) assessed in Fig R15 for PKCζ binding are indicated below. A close-up view of the switchII/III region illustrates the binding sites for RGS proteins and effectors. Important residues for Gαq interaction with PLCβ, GRK2, p63RhoGEF and RGS proteins are highlighted in different colours (data from(Fan et al., 2003; Shankaranarayanan et al., 2010; Tesmer et al., 2005; Tsuboi et al., 2008)). The region corresponding to the C-terminus of the β4 strand and the β4-α3 loop presents a striking sequence similarity to the PB1 domain type I of MEK5, which is known to interact with PKCζ PB1 type II domain. Homologous residues in Gαq with acidic properties were selected for point mutagenesis.

e. Mutation of E234 and E245 in Gαq abrogates PKCζ binding

According to information in Figure R16, we proceeded to mutate negatively-charged residues in the Gαq pseudo-PB1 domain. Gαq mutants were overexpressed in COS-7 cells together with wild-type HA-PKCζ and immunoprecipitation against the HA tag was performed. Most representative results are shown in Fig. 17A. The combined mutation E234/E245-AA in Gαq decreased association with PKCζ up to 70% (Fig. R17A). Interestingly, these two aminoacids are conserved in other PB1-type I domain-harbouring proteins

(MEK5, p62, PKCζ and PKCλ) (Fig. R17B). Additionally, their equivalents in PB1-type I domain proteins are crucial for their function and are part of the two conserved clusters of the PB1-type I domain (A1 and A2) (Fig. R17B)(Hirano et al., 2005). Moreover, an alignment between all members of the Gα family members showed that the coexistence of both E234 and E245 is a distinctive feature of the Gαq/11 subfamily (Fig. R17C). Taken together, data in figures R16 and R17 indicate that a Gαq region in the effector-binding site that resembles a PB1 type I domain is involved in the interaction with PKCζ, with glutamic acids in positions 234 and 245 playing an essential role.

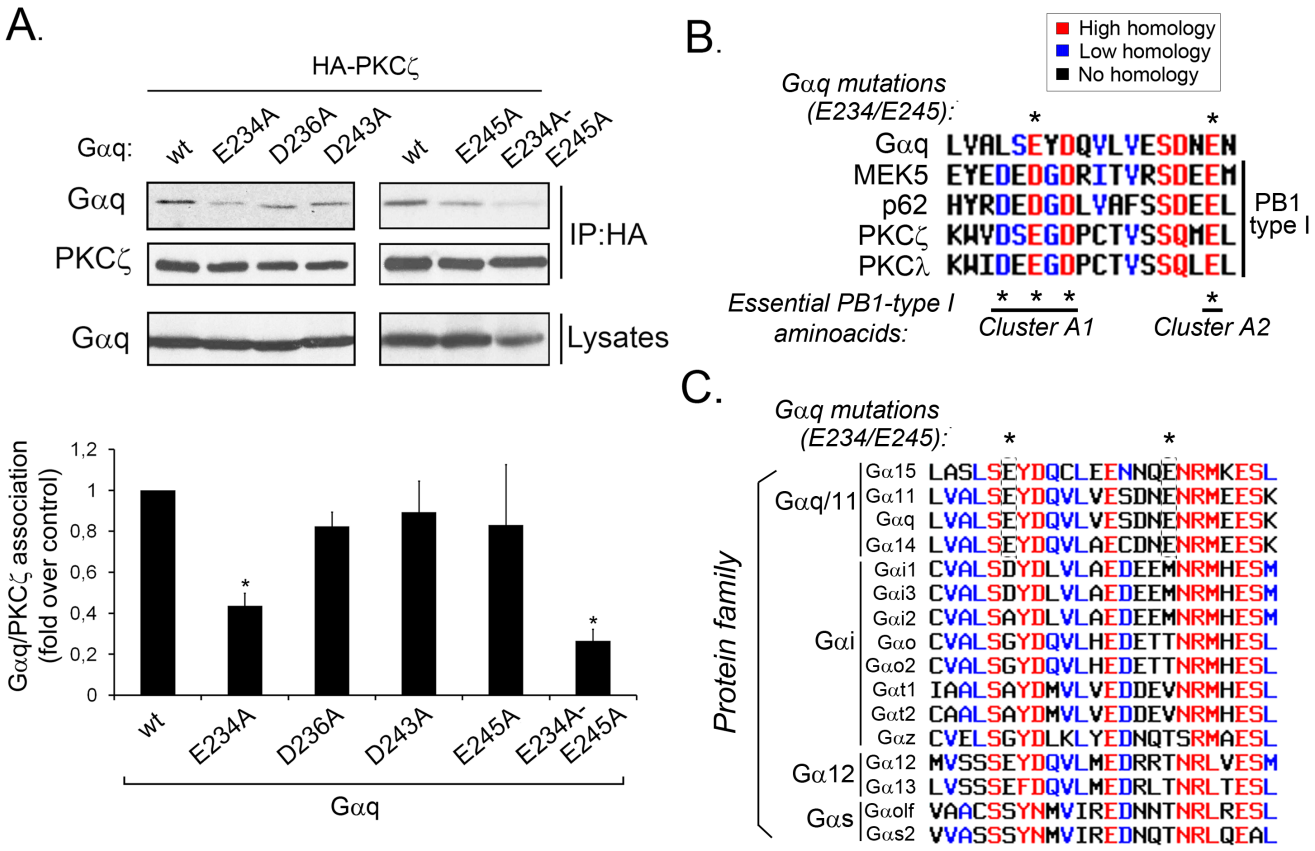

Figure R17. Identification and analysis of crucial aminoacids in Gαq to interact with PKCζ. Alanine-scanning mutagenesis was performed in Gαq as shown in Fig. R16. (A) COS-7 cells were transfected with combinations of plasmids encoding HA-PKCζ and different Gαq mutants. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. To compare the association of PKCζ to the different Gαq mutants, blot bands were quantified and normalised by total HA-PKCζ. Data (mean +/- SEM of three independent experiments) were normalised with respect to PKCζ/Gαq wt association as a control (*p<0.05, two tailed T-test). (B) Sequence alignment of different PB1-type I domains and the β4-α3 loop (pseudo-PB1 domain) of Gαq. Glutamic acids 234 and 245 (E234/E245) of Gαq are conserved in all PB1-type I proteins and are part of the two crucial clusters for their function (A1 and A2). (C) Sequence alignment of the β4-α3 region of different Gα subfamily member. The combined presence of Glutamic acids in positions 234 and 245 is an exclusive feature of the Gαq/11 family.

5. REQUIREMENT OF AN EFFICIENT Gαq/PKCζ ASSOCIATION FOR THE ACTIVATION OF THE ERK5 PATHWAY.

We next sought to establish whether altering the interaction between Gαq and PKCζ affects ERK5 activation. To that purpose, we generated the PKCζ-binding-deficient mutation (E234/E245-AA) in a constitutively activated Gαq background (R183C). We overexpressed GαqR183C with/without the E234/E245-AA mutation and assessed ERK5 activation in HeLa cells (Fig. R18A). The Gαq-R183C-E234/E245-AA mutant was unable to promote ERK5 phosphorylation compared to Gαq-R183C, consistent with the notion that the formation of the Gαq/PKCζ complex is strictly required for ERK5 activation by Gαq. In order to further confirm this, we took advantage of the Gαq-biased properties of the pathway in which the overexpression of Gαq potentiates ERK5 activation by GPCR (see Fig R8). Thus, we overexpressed empty vector (pcDNA3) and either wild-type or PKCζ-binding-deficient forms of Gαq in Cho-M3 cells and stimulated with carbachol to assess the activation of ERK5 (Fig. R18B). Whereas ERK5 phosphorylation was greatly enhanced by wild-type Gαq overexpression, it was completely unaffected by the Gαq E234/E245-AA mutant to the same degree as by empty vector overexpression.

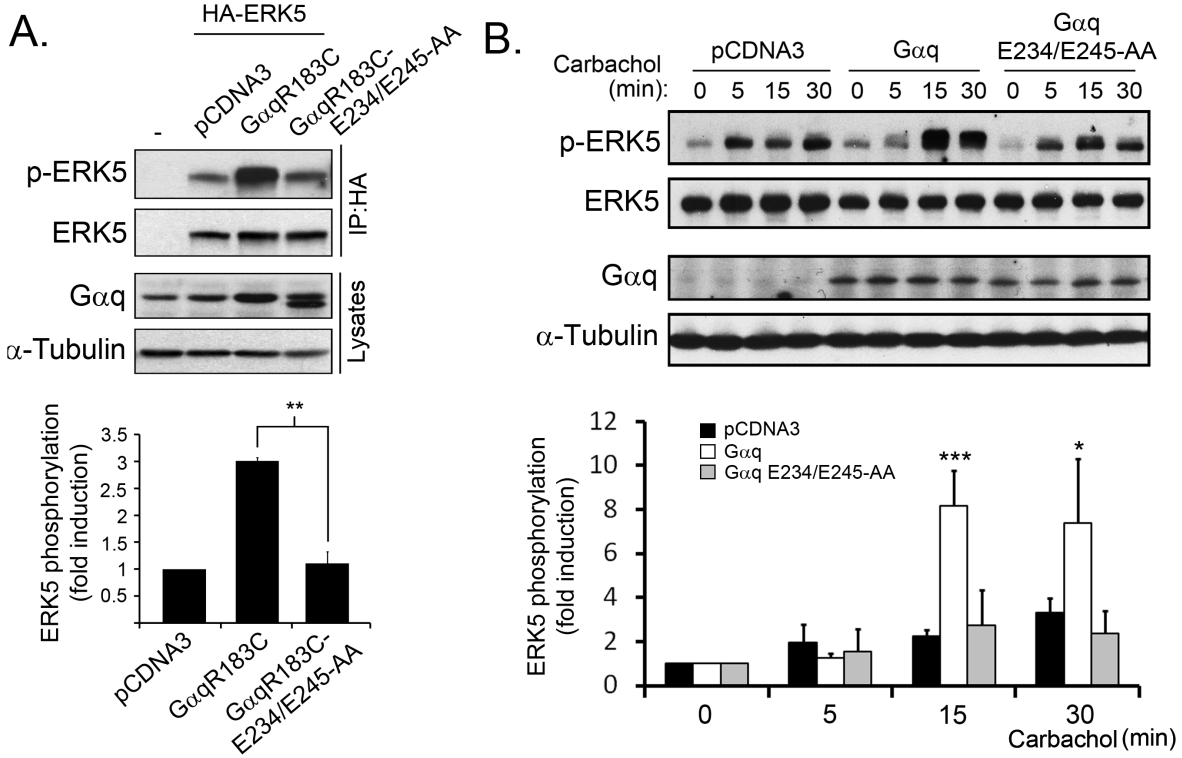

Figure R18. ERK5 activation by Gαq is abolished by the E234/E245-AA mutation. (A) HeLa cells were transfected with different combinations of plasmids encoding HA-ERK5 and the constitutively active mutants GαqR183C and GαqR183C-E234/E245-AA. 24hrs after transfection, cells were lysed and ERK5-HA was immunoprecipitated as previously described. Immunoprecipitates and total lysates were analysed by western blot. Data (mean +/- SEM of 3 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction of ERK5 phosphorylation over control conditions (**p<0.005, two tailed T-test). (B) Cho-M3 cells were transfected with different combinations of plasmids. 24hrs after transfection, cells were serum-starved for 2h and stimulated with carbachol (10μM) for the indicated times. Samples were processed and analysed as in (A) (*p<0.05, ***p<0.001, two tailed T-test). Gαq and alpha-tubulin lysate levels were also determined as expression controls.

To rule out the possibility that the Gαq E235/E245-AA mutant displayed general, non-specific functional defects, we assessed whether this mutation affects coupling to PLCβ and other signalling pathways. Constitutively active forms of Gαq with or without the E234/E245-AA mutation were transfected into HEK293 cells and total inositol phosphate (IP) production was measured. Both constructions significantly increased IP production with respect to empty vector, although the constitutively active form of Gαq without the E235/E245-AA mutation was more efficient (Fig. R19A). This suggests that the E234/E245-AA mutation partly affects PLCβ coupling to Gαq, though it is still able to trigger significant IP production. In this regard, we also assessed the activation of ERK1/2, a primary downstream target of the PLCβ pathway. HEK293 cells were transfected with constitutively active forms of Gαq with/without the PKCζ-association-impairing mutation and ERK1/2 phosphorylation was assessed (Fig. R19B). Results clearly show that both Gαq forms potently enhanced ERK1/2 phosphorylation in a similar fashion, indicating that our mutant does not display defects in its global functionality.

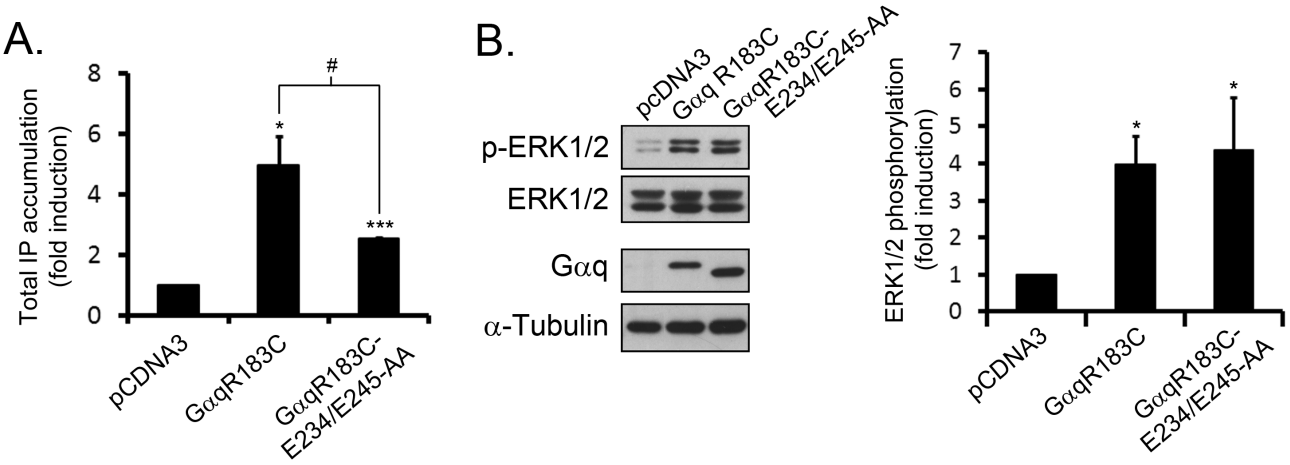

Figure R19. Functional selectivity of the Gαq E234/E245-AA mutant. The ability of the Gαq E234/E245-AA to initiate classic signalling downstream of Gαq was assessed. (A) HEK293 cells were transfected with empy vector (pcDNA3) or plasmids encoding the constitutively active mutants GαqR183C or GαqR183C-E234/E245-AA. Twenty four hours after transfection cells were incubated overnight with titriated inositol. Total inositol phosphate content was determined as detailed in the Methods section. Data (mean +/- SEM of three independent experiments) were normalised to transfection of empty vector (* and # $p<0.05$, ***$p<0.001$ two tailed T-test) (B) HEK293 cells were transfected with empy vector (pcDNA3) or plasmids encoding the constitutively active mutants GαqR183C or GαqR183C-E234/E245-AA. Twenty four hours after transfection cells were lised and ERK1/2 activation was assessed by western blot with specific phospho-antibodies. Data (mean +/- SEM of three independent experiments) were normalised to pcDNA3 transfection (*$p<0.05$, two tailed T-test). Gαq and alpha-tubulin lysate levels were also determined as expression controls.

Overall, we can conclude that a Gαq mutant with impaired ability to associate with PKCζ (Gαq E234/E245-AA) is completely unable to promote either direct (Fig. R18A) or GPCR-initiated (Fig. R18B) activation of ERK5. This strongly suggests that the Gαq/PKCζ complex is strictly required for the activation of ERK5 by Gq-coupled receptors.

6. GRK2 REGULATES Gαq/PKCζ ASSOCIATION AND ERK5 ACTIVATION.

Desensitisation and signal termination processes are essential self-limiting mechanisms of cells to control the duration and amplitude of a given response. For Gαq, two main modulation mechanisms have been described: GTPase activity enhancement by RGS2/4 proteins and sequestering from effectors by GRK2. Thus, we aimed to characterise whether these processes were affecting the Gαq/PKCζ complex formation and in ERK5 activation.

a. RGS proteins do not compete with PKCζ for the same binding site in Gαq

RGS proteins are known regulators of Gα function through the promotion of Gαq GTPase activity. Additionally, they have been reported to bind to the activated G protein without interfering with effector binding. In order to establish whether this was also true for PKCζ, we coexpressed Gαq and HA-tagged PKCζ with or without RGS4 and immunoprecipitated with an anti HA antibody (Fig. R20A). The formation of the Gαq/PKCζ complex was not affected by overexpression of RGS4, suggesting that effector and regulator can simultaneously bind to the G protein. To further confirm this, a similar experiment was carried out with RGS2 (Fig. R20B). The presence of PKCζ in the coimmunoprecipitate was not altered by overexpression of RGS2. Therefore, the two main RGS regulators of Gαq (RGS2 and 4) do not compete with PKCζ for binding. A three-dimensional Gαq model shows that the non-overlapping spatial localization of the RGS-binding region and the pseudo-PB1 domain is consistent with the possibility of simultaneous binding (Fig. R20C) and the occurrence of a ternary complex including effector, Gαq and regulator. Additionally, these data are also consistent with the results for the Gαq/i1 chimeras (Fig. R15) showing that the removal of the RGS binding region (located upstream of the insertion site at position 222) did not affect PKCζ binding (see Fig. R15 and Fig. R16 for graphical explanation).

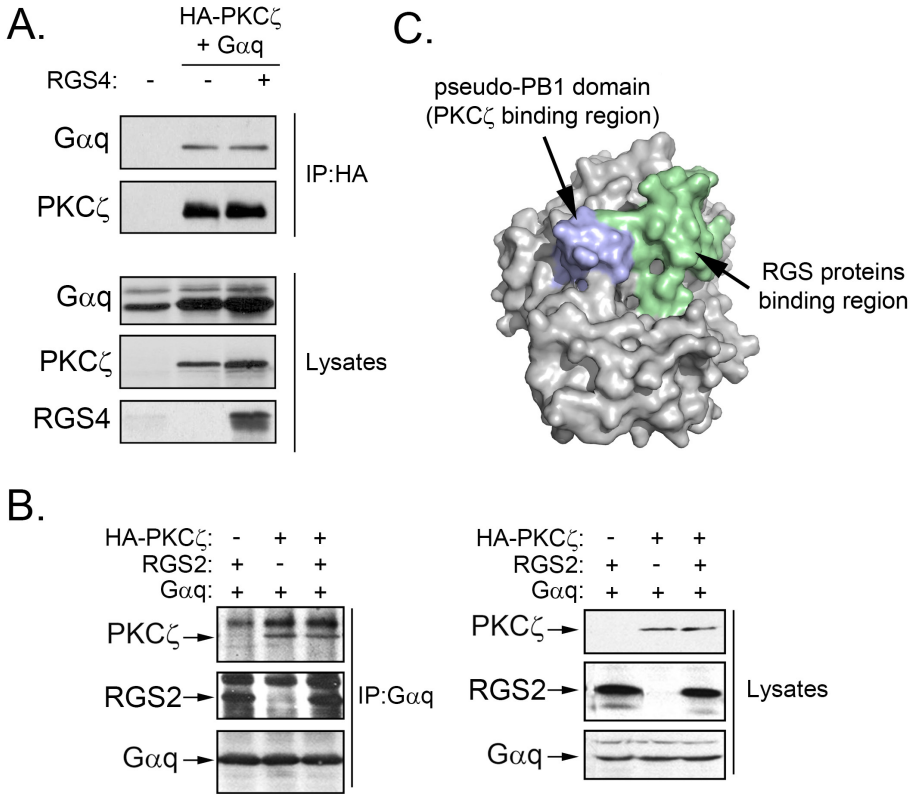

Figure R20. RGS proteins 2 and 4 do not compete with PKCζ for binding to Gαq. COS-7 cells were transfected with combinations of plasmids encoding HA-PKCζ, Gαq and either RGS4 (A) or RGS2 (B). HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads in (A) and Gαq was immunoprecipitated in (B). Immunoprecipitates and total lysates were analysed by western blot with specific antibodies to determine the effect of RGS protein overexpression on the Gαq/PKCζ complex. Blots shown are representative of 3 independent experiments. (C) Gαq crystal structure was modelled from the Gαq-GRK2-Gβγ crystal structures using Pymol software. Different binding regions are highlighted in green [switch I&II, GRS proteins binding region] and blue [β4-α3 region of Gαq, pseudo-PB1 domain (FLVALSEYDQVLVESDENEN), PKCζ binding region].

b. GRK2 prevents both PKCζ and MEK5 to interact with Gαq

GRK2 has been shown to negatively regulate binding of Gαq to effectors and to dampen downstream signalling. We sought to determine whether this negative regulation link was also observed for the components of the Gαq-ERK5 pathway. To that purpose, we overexpressed Gαq and GRK2 together with either PKCζ (Fig. R21A) or MEK5 (Fig. R21B), and Gαq was immunoprecipitated. Analysis of the coprecipitates showed that GRK2 completely abolished Gαq association to either effector (Fig. R21A&B). The competition with PKCζ was further characterised and demonstrated not to depend on the kinase activity of GRK2, since a catalytically inactive mutant (GRK2 K220R) still competes with PKCζ (Fig. R21A). Indeed, the negative effect of GRK2 on Gαq-PKCζ association was found to be solely dependent on its RH domain, a region previously reported to interact with Gαq (Sterne-

Marr et al., 2003) (Fig. R21A). The analysis of the binding regions of PKCζ and GRK2 on the Gαq crystal structure (Tesmer et al., 2005) suggest that although GRK2 and PKCζ do not bind the same subset of aminoacids in Gαq, the binding surfaces are close enough to compete sterically (Fig. R21C).

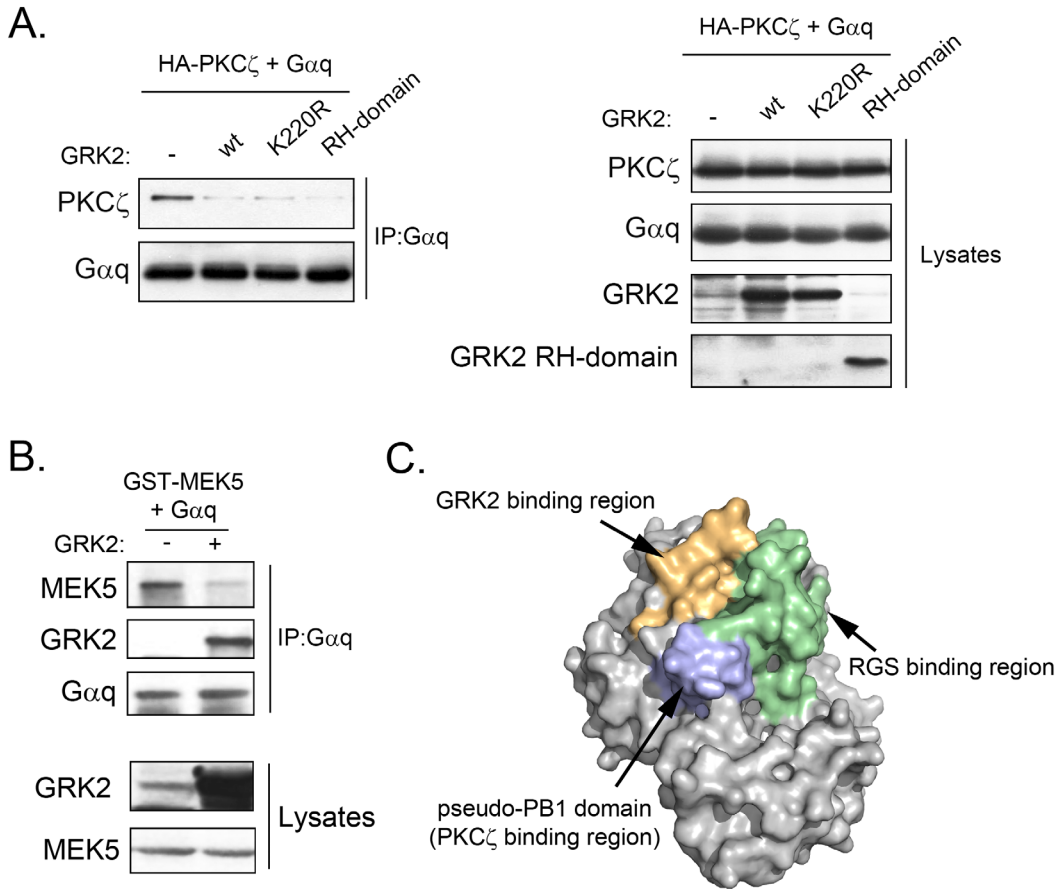

Figure R21. GRK2 is a negative regulator of Gαq association with PKCζ and MEK5 (A) COS-7 cells were transfected with combinations of plasmids encoding HA-PKCζ and Gαq with GRK2 wild type, GRK2 K220R (kinase-dead mutant) or the GRK2 RH domain. Gαq immunoprecipitates and total lysates were analysed by western blot with specific antibodies to determine the effect of the overexpression of GRK2 variants on the Gαq/PKCζ complex. Blots shown are representative of 3 independent experiments. (B) COS-7 cells were transfected with combinations of plasmids encoding GST-MEK5, Gαq and GRK2 and Gαq immunoprecipitates and total lysates analysed to determine the effect of the overexpression of GRK2 variants on the Gαq/MEK5 complex. Blots shown are representative of 3 independent experiments. (C) Gαq crystal structure was modelled from the Gαq-GRK2-Gβγ crystal structures. Different binding regions are highlighted in green [switch I&II, RGS proteins], blue [pseudo-PB1 domain (FLVALSEYDQVLVESDENEN), PKCζ] and orange (switch III (KALFRTIITYPWF), GRK2).

c. GRK2 is a negative regulator of the Gαq/PKCζ complex in living cells

In order to address whether the effect of GRK2 was also detected in a natural cell milieu, a venus YFP PCA-based approach was performed. The Gαq/PKCζ interaction can be detected through this technique as demonstrated in Fig. R10. We overexpressed Gαq and PKCζ fused to cognate fragments of venus-YFP in HEK93 cells and co-expressed either empty vector (pcDNA3) or GRK2. As clearly seen in the microscopy images (Fig. R22A) and in the fluorescence quantification (Fig. R22B), GRK2 significantly decreases the formation of the Gαq/PKCζ complex. The expression of the different constructs was also assessed in Fig. R22C, which showed equal expression of the PCA plasmids upon coexpression of GRK2.

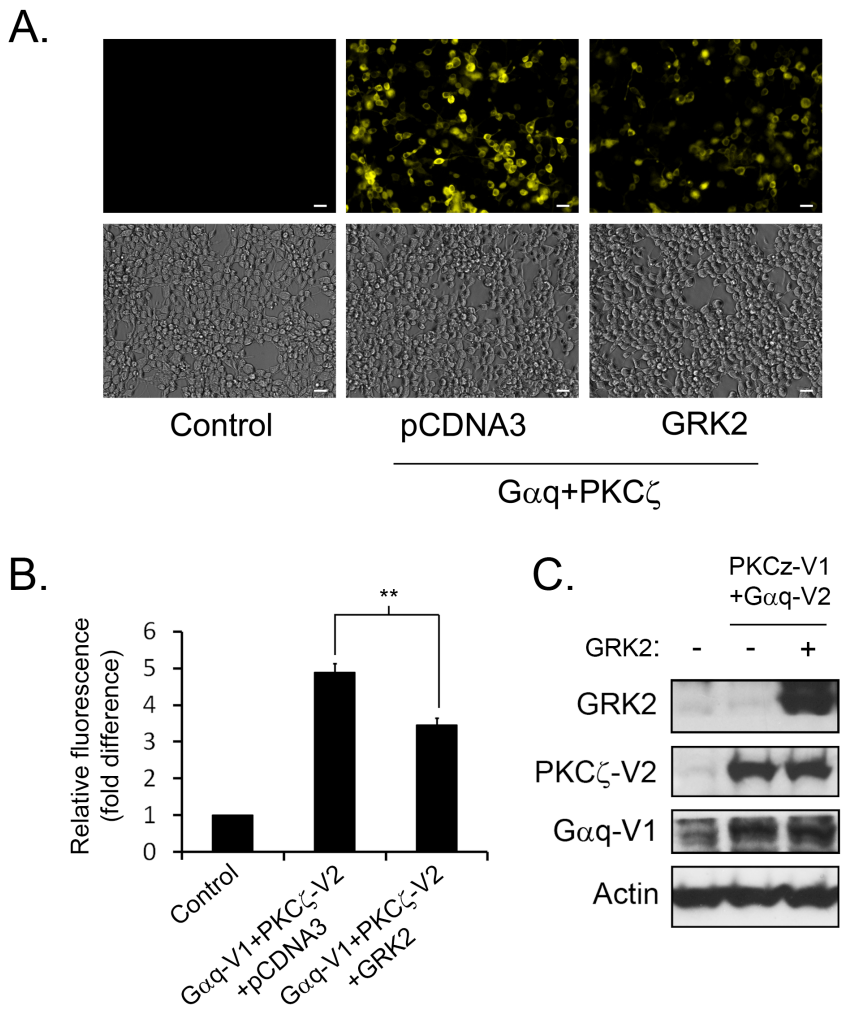

Figure R22. GRK2 is a negative regulator of the Gαq-PKCζ complex in living cells. (A) HEK293 cells were transfected with different pairs of protein complementation assay (PCA) plasmids that express protein pairs fused to cognate fragments of venus-YFP: Control (PKCζ-Venus YFP[F1]+pcDNA3), Gαq+PKCζ+pcDNA3 (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]+pcDNA3), Gαq+PKCζ+GRK2 (Gαq-Venus YFP[F1]+ PKCζ-Venus YFP[F2]+GRK2). Twenty-four hours after transfection cells were visualised under the microscope as detailed in the Methods section. (B) Fluorimetric analysis was performed in order to determine the effect of GRK2 on the PKCζ-Gαq complex as detailed in the Methods section. Data (mean +/- SEM of three independent experiments) were normalised with respect to control. (C) The different cell populations were lysed and analysed by western blot to determine the correct expression of the different plasmids. Specific antibodies were utilised against PKCζ, Gαq, GRK2 and actin as a loading control. Blots shown are representative of 3 independent experiments.

d. Disruption of the GRK2-Gαq complex upregulates PKCζ-Gαq association

We have shown that the RH-domain of GRK2 is sufficient to inhibit the formation of the Gαq/PKCζ complex. Since this is the domain that has been reported to interact with Gαq, we hypothesised that the negative effect of GRK2 was due to a direct binding of GRK2 and Gαq. In order to establish this, we utilised a GRK2 mutant (D110A) that does not interact with Gαq (Sterne-Marr et al., 2003) and assessed the effect of its overexpression on the Gαq/PKCζ complex. Whereas GRK2 wild-type completely abrogated Gαq coimmunoprecipitation with PKCζ, the presence of the GRK2-D110A mutant barely interfered with the formation of the complex (Fig. R23A). Additionally, we utilised two Gαq mutants that are unable to interact with GRK2 (Y261F and W263D)(Tesmer et al., 2005) and assessed their PKCζ-association ability (Fig. R23B). Surprisingly, these Gαq mutants co-immunoprecipitated with PKCζ to a much greater extent. This seems to indicate that a lack of negative regulation by GRK2 on Gαq favours the formation of the Gαq/PKCζ complex. Taken together, these data indicate that GRK2 negatively regulates the Gαq/PKCζ interaction through a direct association with Gαq.

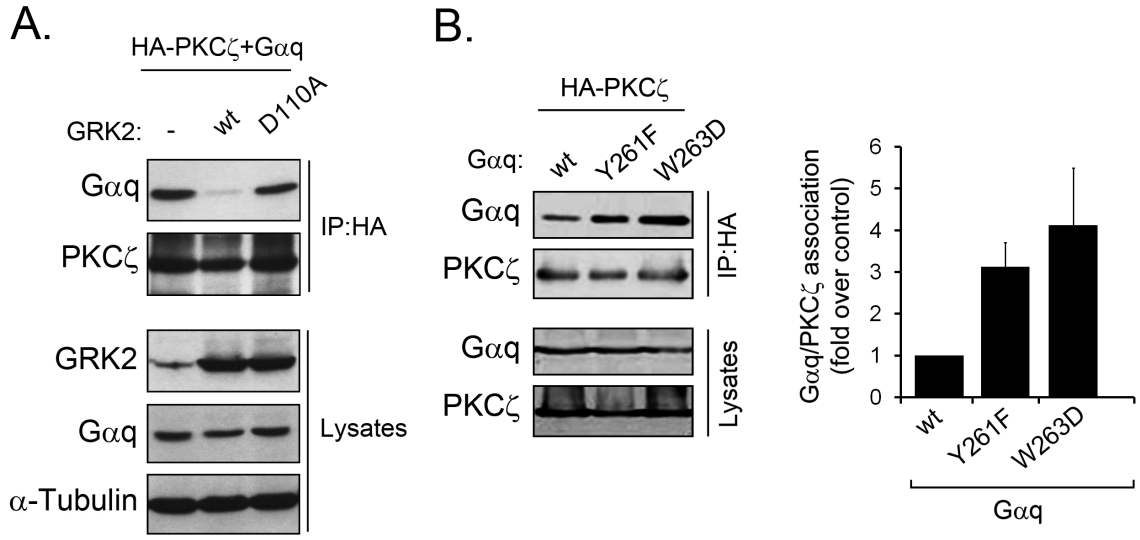

Figure R23. Modification of the GRK2-Gαq interaction surface modulates PKCζ-Gαq association. (A) COS-7 cells were transfected with combinations of plasmids encoding Gαq, HA-PKCζ, GRK2 wt and the GRK2 D110A mutant, which has impaired ability to associate to Gαq. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot to compare the association of Gαq to PKCζ in the presence of the different GRK2 variants. (B) COS-7 cells were transfected with combinations of plasmids encoding HA-PKCζ and Gαq mutants deficient in association to GRK2 (GαqY261F and GαqW263D). HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. To compare the association of PKCζ to the different Gαq mutants, blot bands were quantified and normalised by total HA-PKCζ. Data (mean +/- SEM of 3 independent experiments) were normalised with respect to PKCζ/Gαq wt association as a control. Blots shown are representative of 3 independent experiments.

e. ERK5 activation by Gq-coupled GPCR is negatively regulated by GRK2

Since GRK2 negatively regulates association of Gαq with both PKCζ and MEK5, we hypothesised that the disruption of this interaction would impair the activation of ERK5. In order to establish that, we transfected Cho-M3 cells with either empty vector (pcDNA3) or GRK2 and assessed ERK5 activation by carbachol at different times (Fig. R24A). The results clearly show that the overexpression of GRK2 completely abolishes ligand-induced ERK5 phosphorylation. The negative effect of GRK2 on Gαq signalling pathways have been tied to receptor phosphorylation and desensitisation, and also to sequestering of activated Gαq. Thus, we assessed whether a catalytically inactive mutant (GRK2 K220R) and a Gαq interaction-deficient mutant (GRK2 D110A) would differentially downregulate the ERK5 pathway. We transfected cells with either GRK2 wild-type/K220R/D110A and assessed ERK5 maximal activation by carbachol at 15 min (Fig. R24B). It was observed that both mutants, and specially D110A, display a significantly reduced ability to downregulate the ERK5 pathway compared to wild-type. Thus, it seems that both receptor desensitisation and Gαq sequestering are necessary processes for ERK5 pathway downregulation by GRK2.

In order to further characterise the role of GRK2 in directly binding and sequestering Gαq away from effectors, we studied the effect of the RH domain of the kinase on ERK5 activation by carbachol. This domain binds to Gαq and is responsible for the inhibitory effect on the G protein, and also we have observed that it is sufficient to displace PKCζ from a complex with Gαq (Fig R21a). As shown in Fig. R24C, the overexpression of the RH domain accounted for approximately half of the inhibitory effect of GRK2 on ERK5 activation. This is consistent with the notion that ERK5 activation is inhibited by a combined effect of GRK2 on the receptor and on Gαq through its RH domain. Finally, since we have seen that a lack of regulation by GRK2 on Gαq upregulates the Gαq/PKCζ complex (Fig R22b), we hypothesised that this would be accompanied by an upregulation of the activation of ERK5. Thus, we compared the activation of ERK5 by carbachol in Cho-M3 cells transfected with either Gαq wild-type or Gαq Y261F (GRK2 binding-deficient mutant) (Fig. R24D). As the results clearly show, Gαq Y261F mutant greatly enhances the duration and amplitude of ERK5 activation by carbachol compared to control. Taken together, these data strongly point at GRK2 as a major regulator of the Gαq-ERK5 pathway through a negative dual action on the activated receptor and on the formation of efficient G protein-effector complexes.

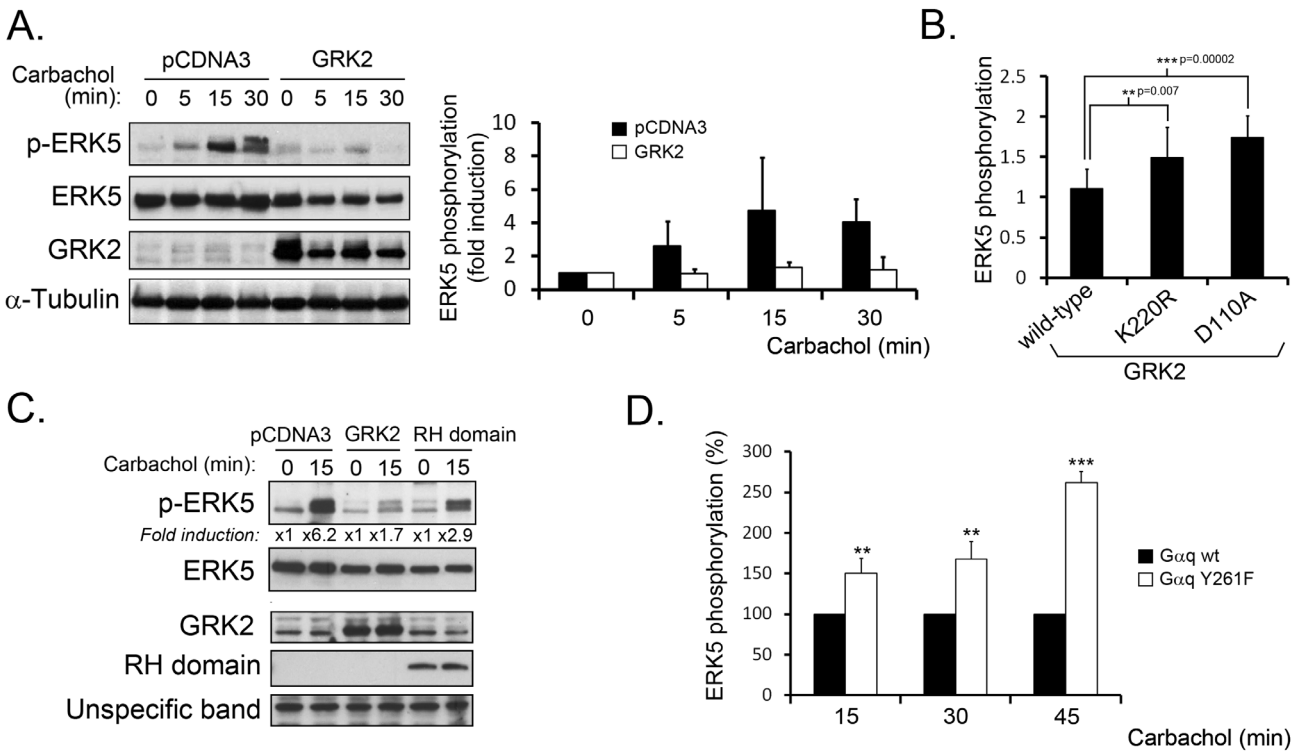

Figure R24. ERK5 activation by Gq-coupled GPCR is negatively regulated by GRK2. GRK2 abolishes ERK5 activation by Gq-coupled muscarinic M3 receptor through both receptor phosphorylation and inhibition of Gαq-effector interactions. (A) Cho-M3 cells were transfected with different combinations of empty vector (pcDNA3) or plasmids encoding HA-ERK5 and GRK2. Twenty-four hours after transfection, cells were serum-starved for 2h and stimulated with carbachol (10μM) for the indicated times. Cells were lysed and ERK5-HA was immunoprecipitated with an anti-HA agarose-conjugated antibody as detailed in the Methods section. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. Data (mean +/- SEM of 3 independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction of ERK5 phosphorylation. (B) Cho-M3 cells were transfected with HA-ERK5 and GRK2 wt, GRK2 K220R (kinase-dead mutant) or GRK2 D110A (Gαq-interaction deficient mutant). 24 hours after transfection cells were serum starved and stimulated with carbachol (10μM) for 15 min. Samples were processed as above (two-tailed T-test with indicated significance). (C) Cho-M3 cells were transfected with HA-ERK5 and pcDNA3, GRK2 wt or the GRK2-RH domain. Samples were processed as above. Blots shown are representative of 2 independent experiments. (D) Cho-M3 cells were transfected with HA-ERK5 and either Gαq wt or Gαq Y261F. 24 hours after transfection cells were serum starved and stimulated with carbachol (10μM) for the indicated times and processed as above. Data (mean +/- SEM of three independent experiments) were normalised using ERK5 as loading control and expressed as fold-induction of carbachol-induced ERK5 phosphorylation upon Gαq wild type overexpression at each time point (**p<0.005, ***p<0.001, two tailed T-test).

7. ACTIVATION MECHANISMS OF THE Gαq/PKCζ/ERK5 PATHWAY

During this work, we have put forward PKCζ as an essential effector of Gαq in the activation of ERK5 by Gq-coupled GPCRs. Extensive work has been done in order to dissect the different protein-protein interactions that are involved in the Gαq/PKCζ/ERK5 pathway but the activation mechanisms operating still remain to be elucidated.

a. PKCζ is not phosphorylated on T410 or T560 upon Gq-coupled GPCR stimulation

PKCζ is a Ser/Thr protein kinase with different activation mechanisms (see Introduction, Fig. I7). The most commonly described ones involve the trans-phosphorylation of its activatory loop on Thr410 and the autophosphorylation of Thr560. Since we have described that ERK5 activation by Gq-coupled GPCRs seems to require PKCζ activity as indicated by the consistent inhibition of the pathway by the pseudosubstrate catalytic inhibitor (Fig R2b, R3b and García-Hoz et al., 2010), we hypothesised that the kinase could be activated through phosphorylation-dependent mechanisms. Thus, we stimulated NIH-3T3-M1 cells with carbachol at different times and assessed the phosphorylation status of endogenous PKCζ on both Thr410 and Thr560 (Fig. R25). Although PKCζ seems to be basally phosphorylated on both residues, no modulation can be ascribed to carbachol addition. This result suggests that PKCζ is not activated by Gq-GPCR through phosphorylation.

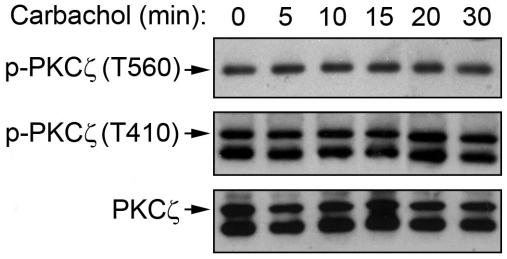

Figure R25. PKCζ is not phosphorylated on T410 or T560 upon by Gq-GPCR stimulation. NIH-3T3-M1 cells were serum-starved for 5h and stimulated with carbachol (10μM) for the indicated times. Cells were lysed and analysed by western blot with specific antibodies against phospho-threonines 410 and 560. Antibodies against total PKCζ and phospho410 also recognise PKCλ, hence the double banding. PKCζ corresponds to the band of slower electrophoretic mobility. Blots shown are representative of 3 independent experiments.

b. PKCζ transiently dimerises in response to Gq-coupled GPCR stimulation

PKCζ has been previously shown to be activated independently of phosphorylation (see Introduction section 3b and Fig. I7). Indeed, a recently proposed mechanism involves catalytic activation due to a PB1-PB1 interaction with Par6 that releases the pseudosubstrate inhibitory domain from the active site of PKCζ. Since PKCζ also harbours a highly homologous PB1 domain to the one in Par6 (PB1-type II domain), we hypothesised that activation could also occur as a result of dimerisation events between different PKCζ molecules through their PB1 domains (type I and II). Thus, we initially set out to investigate whether PKCζ was able to form dimers in living cells. We performed a venus-YFP-based PCA with a pair of plasmids that encode for PKCζ tagged to either N- or C-terminal halves of YFP and expressed them in Cho-M3 cells. Fluorescence visualisation by microscopy and quantification show that PKCζ readily dimerises into high affinity complexes compared to Zipper dimer formation (Fig. R26A). In order to assess whether PKCζ dimerisation was modulated by Gq-GPCR stimulation, we performed a Renilla luciferase-based PCA assay. This approach was developed to study the dynamics of protein-protein interactions as the formation of complexes is reversible (Stefan et al., 2007). The detection of protein-protein interaction relies on the reconstitution of the enzyme Renilla luciferase that converts the substrate benzyl-coelenterazine into a bioluminescent product (Fig. R26B). Thus, bioluminescence is an indirect measure of protein-protein interactions. We carried out this assay by overexpressing PKCζ tagged to N- and C-terminal halves of the enzyme in Cho-M3 cells, stimulated with carbachol and measured bioluminiscence at different times. As shown in Fig. R26C, carbachol promoted a clear and transient increase of PKCζ dimerisation compared to control. Taken together, these data show that PKCζ forms high-affinity dimers in living cells, and that this process can be positively modulated by carbachol. Dimerisation might therefore provide a mechanism for PKCζ activation by Gq-coupled receptors.

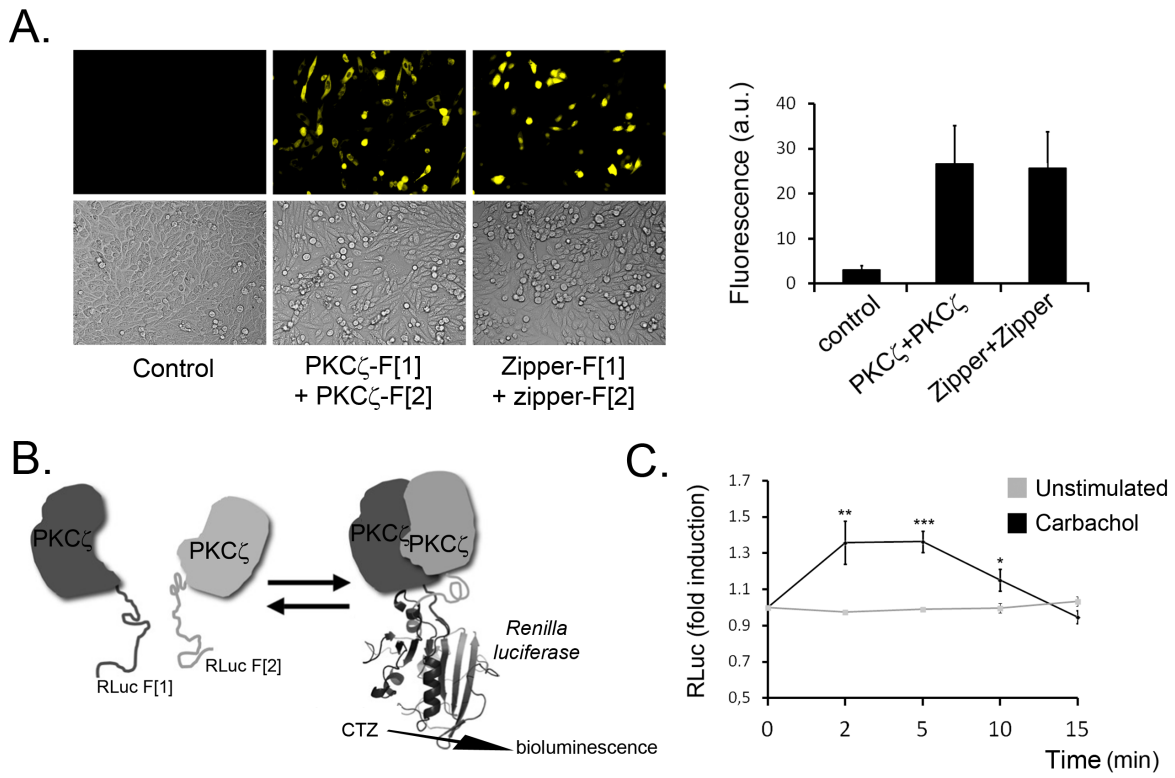

Figure R26. Transient PKCζ dimerisitation is promoted by Gq-coupled GPCR stimulation. (A) Cho-M3 cells were transfected with different pairs of protein complementation assay (PCA) plasmids that express proteins fused to cognate fragments of venus-YFP as indicated in the figure. Twenty-four hours after transfection cells were visualised under the microscope as detailed in the Methods section. Representative brightfield and YFP fluorescence images at 40x magnification are shown. Fluorimetric analysis of the populations was performed and data (mean +/- SEM of three independent experiments) were normalised with respect to control. (B) Conceptual explanation of renilla luciferase PCA, a technique that utilises the reversible reconstitution of a luciferase enzyme from N/C-terminal halves that are fused to two proteins of interest. Bioluminiscence in living cells upon the enzyme´s substrate (CTZ) addition is a measure of protein-protein interactions, which brings cognate fragments of renilla luciferase close enough to allow reconstitution of the native fold. (C) Cho-M3 cells were transfected with PKCζ-Venus YFP[F1] and PKCζ-Venus YFP[F2]. Twenty-four hours after transfection, cells were serum-starved for 2h and stimulated with carbachol (10μM). Bioluminiscence was measured at 482 nm as detailed in the Methods section and data (mean +/- SEM of 3 independent experiments) were normalised with respect to control (**p<0.005, ***p<0.001, two tailed T-test).

c. PKCζ acts as a scaffold between Gαq and ERK5 upon GPCR activation

The finding that PKCζ dimerises in response to carbachol poses new questions with regards to the formation of different protein complexes in the activation of ERK5. Indeed, the concentration of PKCζ dimers/oligomers within specific cellular locations might provide a signalling platform for the association of the different molecules involved in the pathway. This scaffold role, driven by PB1 domains, has been widely documented for other processes (see Introduction, section 3b and fig. I9). It has previously been shown by us and others that PKCζ interacts directly with both Gαq and MEK5 (García-Hoz et al., 2010; Diaz-Meco & Moscat) in the activation of ERK5. Additionally, it has recently been demonstrated

that PKCζ directly associates with ERK5 and this interaction involves the kinase domain of PKCζ (Nigro et al., 2010). In order to determine whether a PKCζ/ERK5 complex was also occurring in our system, we studied the association of these two proteins upon carbachol stimulation. Initially, we confirmed that PKCζ and ERK5 coimmunoprecipitated in COS-7 cells (Fig. R27A). Then, HA-PKCζ was transfected into NIH-3T3-M1 cells and carbachol was added for different times. PKCζ complexes were immunoprecipitated with an anti-HA antibody and were checked for the presence of endogenous ERK5. Indeed, ERK5 was detected to coimmunoprecipitate with PKCζ at 5 minutes after carbachol addition (Fig. R27B). Interestingly, this closely coincides with the time-frame of the Gαq/PKCζ association in response to carbachol reported in the same system (García-Hoz et al., 2010). Thus, we investigated whether ERK5 and Gαq were part of the same multimolecular complex through an interaction with PKCζ. We found that wild-type Gαq coimmunoprecipitated with ERK5 (Fig. R27C, lane 2) and this was greatly enhanced when Gαq was active (Gαq-Q209L) (Fig. R27C, lane 3). This indicated an activation-dependent association. Since the PB1 domain of PKCζ acts as a dominant negative on the Gαq/PKCζ complex (see Fig. R12C) we overexpressed this domain in order to determine whether PKCζ was bridging ERK5 and Gαq together. Indeed, upon overexpresssion of the PKCζ-PB1 domain Gαq coimmunoprecipitation with ERK5 was completely abolished (Fig. R27C, lane 4). Taken together, these data suggest that PKCζ is acting as a scaffold between ERK5 and Gαq upon activation GPCR.

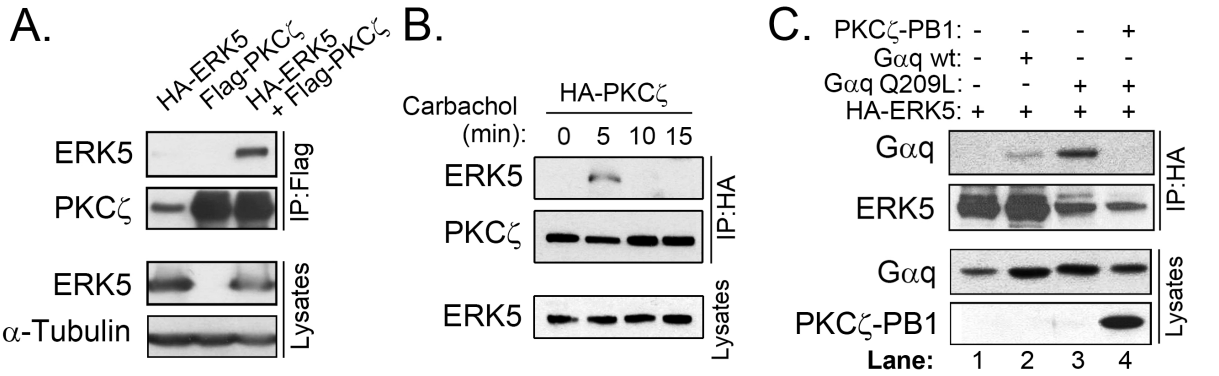

Figure R27. PKCζ interacts with ERK5 upon Gq-GPCR stimulation and brings it into an activated protein complex with Gαq. (A) PKCζ coimmunoprecipitates with ERK5. COS-7 cells were transfected with combinations of plasmids encoding Flag-PKCζ and HA-ERK5. PKCζ was immunoprecipitated with anti-Flag antibody. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies. A representative blot from 3 independent experiments is shown. (B) PKCζ and ERK5 endogenous association is transiently promoted by carbachol. NIH-3T3-M1 cells were transfected with HA-PKCζ. 48h after transfection cells were serum-starved for 5-6 hours and stimulated with carbachol for the indicated times. HA-PKCζ was immunoprecipitated with anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies to assess the coimmunoprecipitation of endogenous ERK5 and PKCζ. Representative blot of 2 independent experiments is shown. (C) Activated Gαq coimmunoprecipitates through PKCζ with ERK5. COS-7 cells were transfected with combinations of plasmids encoding HA-ERK5, Gαq wt, GαqQ209L (constitutively active mutant) and the PKCζ-PB1 domain. HA-ERK5 was immunoprecipitated with an anti-HA antibody coupled to agarose beads. Immunoprecipitates and total lysates were analysed by western blot with specific antibodies to assess the coimmunoprecipitation of ERK5 and Gαq. A representative blot from 3 independent experiments is shown.

8. CELLULAR FUNCTIONS OF THE Gαq/PKCζ COMPLEX

Gαq plays a large number of cellular functions (extensively covered in the Introduction, sections 2b and 5). Since we have established a signalling axis emanating from Gq-coupled muscarinic receptors that requires the formation of the Gαq/PKCζ complex, we sought to determine its role in different cellular processes. To that purpose, we utilised a Gαq mutant with impaired binding to PKCζ (GαqE234/E245-AA; Gαq-EEAA) that was extensively characterised in figures R17, R18 and R19. The general procedure involved comparison of cell proliferation, cell surface, cell adhesion and cell viability between three different populations: Control (empty vector pcDNA3), Gαq (overexpressing Gαq wild-type) and Gαq-EEAA (overexpressing GαqE234/E245-AA). The xCELLigence software was utilised to detect changes in the cell index (a measure of cell attachment to the plate) which can be correlated to proliferation, adhesion and viability changes (see Methods for detailed information).

a. The Gαq/PKCζ complex is involved in carbachol-induced cell blebbing and growth arrest

We aimed to determine the contribution of PKCζ as a Gαq effector in the cellular effects promoted by muscarinic receptor activation. Initially, we analysed the changes in the cell index upon carbachol addition to Cho-M3 cells. The most immediate effect was a drastic and transient increase in the cell index due to cell blebbing, a process that has previously been reported to follow muscarinic receptor stimulation (Street et al., 2006). We observed a clear dose-dependent increase in the slope of the cell index curve compared to control that peaked after 1 hour of carbachol stimulation (Fig. R28A). This indicated that carbachol-induced cell blebbing resulted in an enhanced cell surface coverage and could be readily detected through cell index changes with the xCELLigence software. Next, we compared the blebbing response between control, Gαq and Gαq-EEAA populations. As shown in Fig. R28B, Gαq cells displayed a significantly greater blebbing response compared to control (pcDNA3) indicating that, as previously reported (Street et al., 2006), muscarinic-induced blebbing is Gαq-dependent. Interestingly, Gαq-EEAA cells displayed an overall blebbing response that does not significantly differ from control cells, thus suggesting that PKCζ is involved in Gαq-dependent cell blebbing. If PKCζ is indeed playing a role in the formation of cell blebs through its interaction with Gαq we hypothesised that the Gαq/PKCζ complex could be detected in these cellular protrusions upon carbachol stimulation. Thus, we performed a venus-YFP-based PCA assay to detect the Gαq/PKCζ complex in living Cho-M3 cells. We expressed PKCζ and Gαq fused to cognate fragments of venus-YFP, stimulated with carbachol and monitored the cells for different times. As shown in Fig. R29, cells respond to carbachol addition by emitting different membrane

protrusions (blebs) in a reversible manner. During this process, the YFP fluorescent signal indicative of Gαq/PKCζ association was transiently redistributed and concentrated towards membrane blebs. Taken together, these data suggest that the Gαq/PKCζ pathway plays a relevant role in cell blebbing taking place in response to muscarinic M3 receptor stimulation.

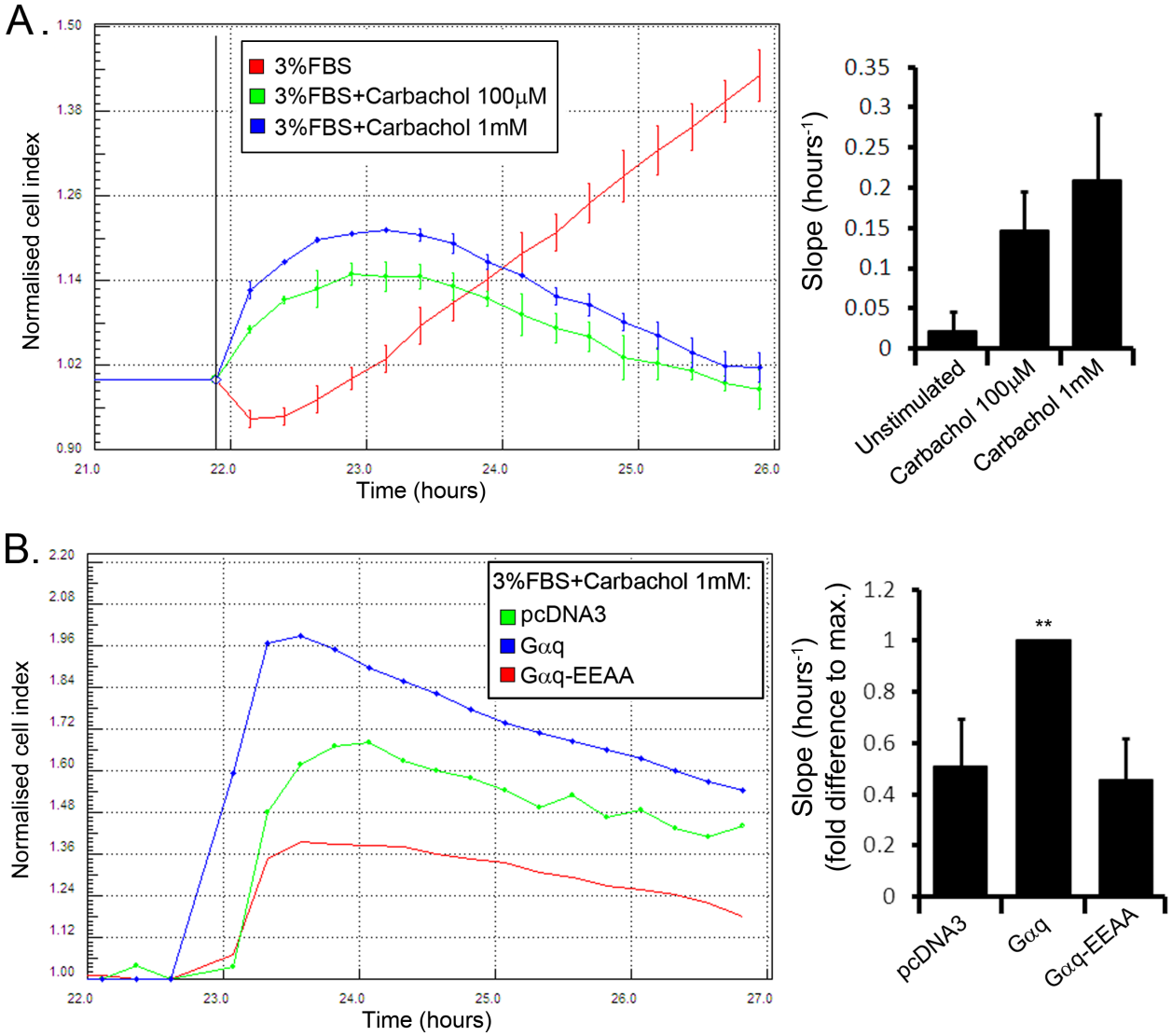

Figure R28. An efficient interaction between Gαq and PKCζ is required for carbachol-induced membrane blebbing. (A) Membrane blebbing is induced by carbachol as detected by the xCELLigence software. Cho-M3 cells were seeded onto 96-well gold electrode sensor plate in 3% FBS and allowed to attach to the plate. After 24hrs, cells were challenged to carbachol stimulation at 100µM and 1mM final concentrations. Monitoring of cell index changes was performed every 15min from the start of the experiment and was continued for a minimum of 6 hours after the treatment. Cell index values were normalised at the time of carbachol addition. Blebbing response was calculated as the slope of the cell index curve between the addition of carbachol until reaching a maximum and was expressed as the mean +/- SEM of 3 independent experiments. (B) Carbachol-induced blebbing is not promoted by the Gαq-E234/E245-AA (Gαq-EEAA) mutant as compared to wild-type Gαq. Cho-M3 cells were transiently transfected with GFP and either pcDNA3 empty vector, Gαq wild-type or Gαq-EEAA. After 24hrs, GFP-positive cells were sorted with a cytometer (see Methods) and were seeded onto 96-well gold electrode sensor plate in 3% FBS. The experiment proceeded as in (A). Blebbing response was calculated as slope of the cell index curve of each population from the addition of carbachol until reaching a maximum. Data (mean +/- SEM of 3 independent experiments) was normalised to the maximum blebbing response corresponding to Gαq-wild type-expressing cells (**p<0.005, two tailed T-test).

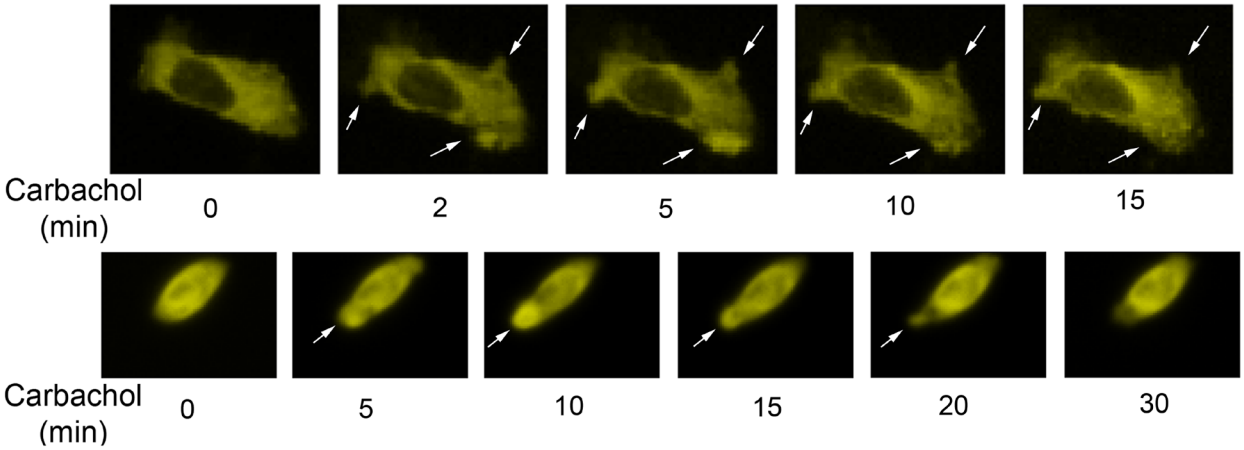

Figure R29. The PKCζ/Gαq complex is localised in membrane blebbing processes upon carbachol stimulation. Cho-M3 cells were transfected with Gαq-Venus YFP[F1] and PKCζ-Venus YFP[F2] plasmids that express proteins fused to cognate fragments of venus-YFP. Twenty-four hours after transfection cells were starved for 2 hours and visualised under the microscope upon carbachol addition for the indicated times, as detailed in the Methods section. Images shown were taken at 40x magnification and subsequently cropped to show single cells. White arrows indicate cell protrusion due to membrane blebbing. Images are representative of 3 independent experiments and show two characteristic morphologies of Cho cells in this experimental setting: a fully-attached cell (above) and a rounded, partially-detached cell (below).

In addition to studying early cell responses to carbachol, we also analysed long-term effect of the ligand. It has been previously reported that M3 muscarinic receptor causes cell growth arrest in Cho cells (Burdon et al., 2002). Indeed, we could reliably detect a dose-dependent growth inhibition by carbachol in 3% fetal bovine serum (FBS) with the xCELLigence software that lasted for a minimum of 24 hours after ligand addition (Fig. R30).

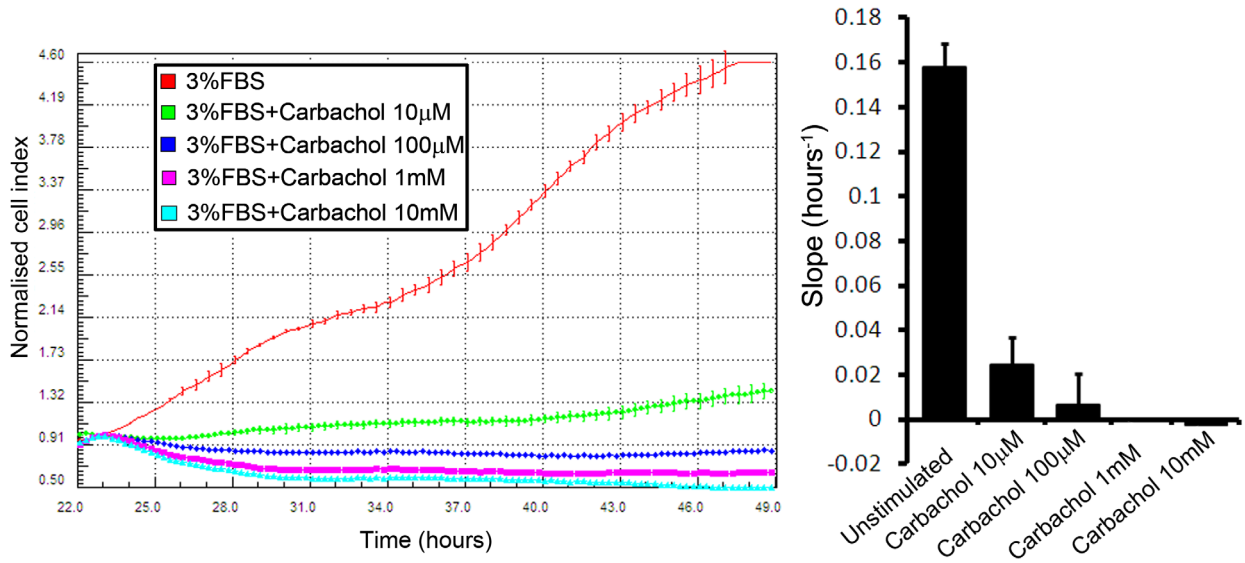

Figure R30. Carbachol induces Cho cell growth arrest. Cho-M3 cells were seeded onto 96-well gold electrode sensor plate in 3% FBS and allowed to attach to the plate. Twenty four hours after plating, cells were challenged with carbachol at various concentrations. Monitoring of cell index changes with the xCELLigence technology was performed every 15min from the start of the experiment and was continued after the treatment. The negative effect of carbachol on cell growth was detected for a maximum of 72h after which time cells invariably started to proliferate again. Cell index values were normalised 6 hours after the addition of carbachol (to exclude the blebbing effect) and cell growth was calculated as the slope (hours-1) of the cell index curve during 24 hours. Slope data was expressed as the mean +/- SEM of 3 independent experiments.

Since we reported in cell blebbing studies that overexpression of Gαq enhances carbachol-induced cellular responses, we compared control, Gαq and Gαq-EEAA populations in the promotion of cell growth arrest by carbachol (Fig. R31). In figure R31A we observe that carbachol inhibits growth in all three populations at two different concentrations (10μM and 1mM) but there is a clear difference in the cell index slope between them. In order to highlight this distinct behaviour, we analysed the decrease in the cell index between control, Gαq and Gαq-EEAA populations at 1mM carbachol (Fig. R31B). Whereas Gαq cells displayed a greater cell growth inhibition, Gαq-EEAA cells did not differ from control. These results suggest that Gαq/PKCζ association is required for muscarinic M3 receptor-induced cell growth inhibition.

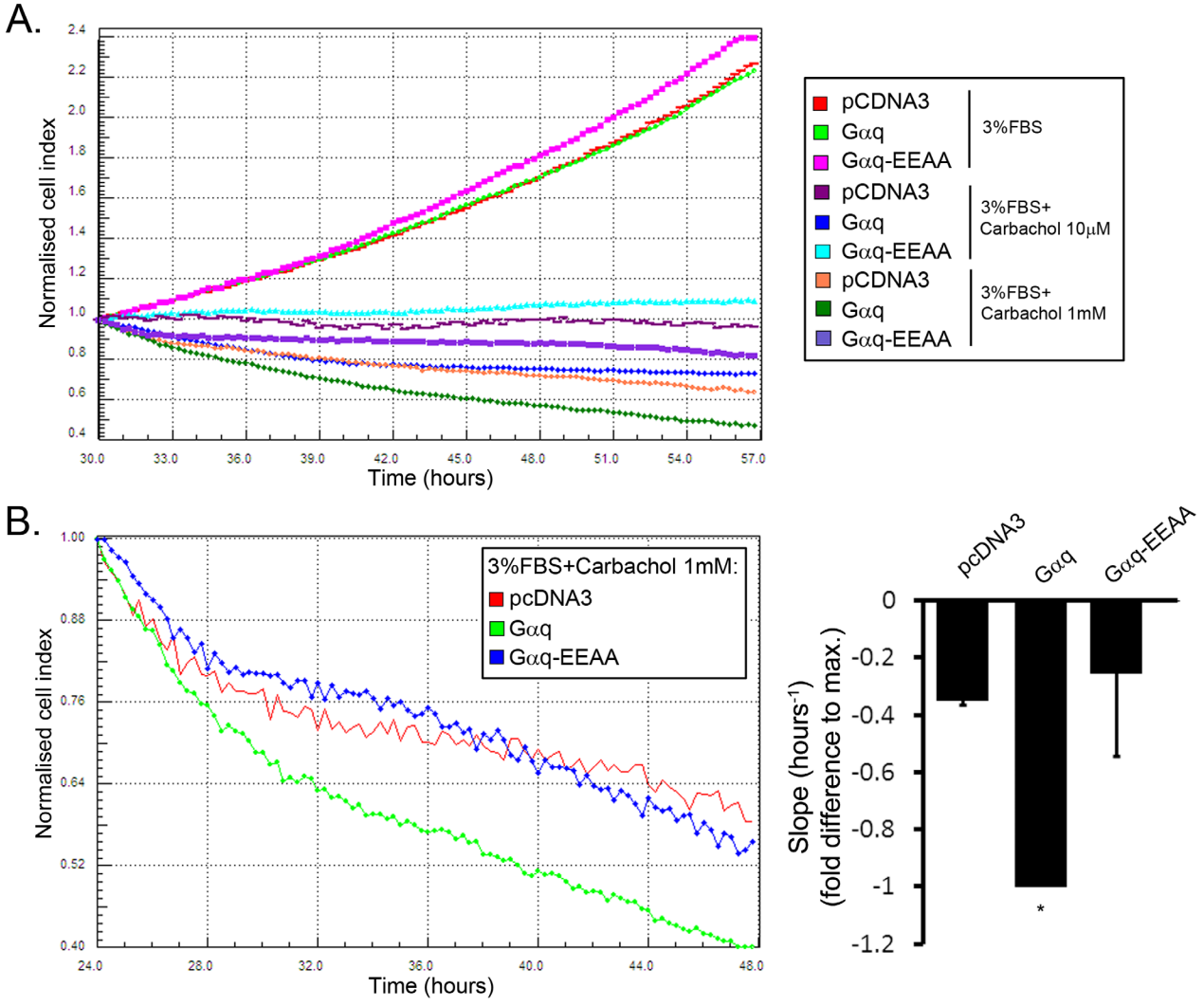

Figure R31. The interaction between Gαq and PKCζ is required for carbachol-induced cell growth arrest. Cho-M3 cells were transiently transfected with GFP and either pcDNA3 empty vector, Gαq wild-type or the Gαq-E234/E245-AA (Gαq-EEAA) mutant. After twenty four hours, GFP-positive cells were sorted with a cytometer to guarantee homogeneous cell populations (see Methods) and seeded onto 96-well gold electrode sensor plate in 3% FBS. The experiment proceeded as in figure R30. The chart above shows the global experiment with two carbachol concentrations and including positive untreated controls. To appreciate differences, treated pcDNA3, Gαq and GαqEEAA populations at 1mM carbachol were amplified below and cell growth was calculated as the cell index increase during 24 hours starting from 6 hours after the addition of carbachol (to exclude the blebbing effect). Data (mean +/- SEM of 3 independent experiments) was normalised to the maximum cell growth arrest effect corresponding to Gαq-wild type-expressing cells (*p<0.05, Two-tailed T-test).

b. The Gαq/PKCζ complex is involved in apoptosis entry

The drastic cell index decrease in Gαq cells stimulated with carbachol (Fig. R31B) suggests the occurrence of apoptosis events in addition to cell growth arrest. This is in agreement with the reported apoptotic response of cells with overstimulated Gαq signalling, especially in the heart (see introduction, section 5a). Additionally, although carbachol-induced cell blebbing is transient and apoptosis-related cell blebbing is irreversible, similar cytoskeletal changes are thought to operate in both processes (Mills et al., 1999). Thus, we were interested in ascertaining whether Gαq and Gαq-EEAA cells differed in their ability to enter apoptosis. To that purpose cells were grown for over 96 hours in 3% FBS and monitored with the xCELLigence software. Apoptosis entry was characterised by a drastic and irreversible decrease in the cell index that occurred after a minimum of 3 days of growth in minimal medium. As shown in Fig. R32A, Gαq cells displayed a significantly anticipated apoptosis entry compared to control cells. Interestingly, Gαq-EEAA cells entered apoptosis at similar times to control cells suggesting that the impairment in the Gαq/PKCζ complex abrogates Gαq-promoted apoptosis. Inspection of the cells through microscopy after approx. 130 hours of growth, at which time Gαq cells but not control or Gαq-EEAA cells had entered apoptosis, show clear differences in cell morphology (Fig. R32B). Indeed, blebbing processes are distinctively detected in Gαq cells but not in control or Gαq-EEAA cells supporting the different apoptotic properties of each population. Since our data suggest that the mutant Gαq-EEAA had a reduced ability to enter apoptosis, we sought to determine whether this was solely caused by a lack of interaction with PKCζ. To that purpose we studied apoptosis entry upon pharmacological inhibition of PLCβ (Fig. R32C). If PLCβ is involved in this process, the addition of the inhibitor would balance the differences between the apoptotic behaviour of Gαq and Gαq-EEAA cell populations. However, as clearly shown in Fig. R32C, the difference in apoptosis entry times was conserved upon PLCβ inhibition, which suggests that PLCβ-downstream pathways are not responsible for the differences observed. Further work needs to be carried out to clearly establish the mechanisms of Gαq/PKCζ-dependent apoptosis promotion.

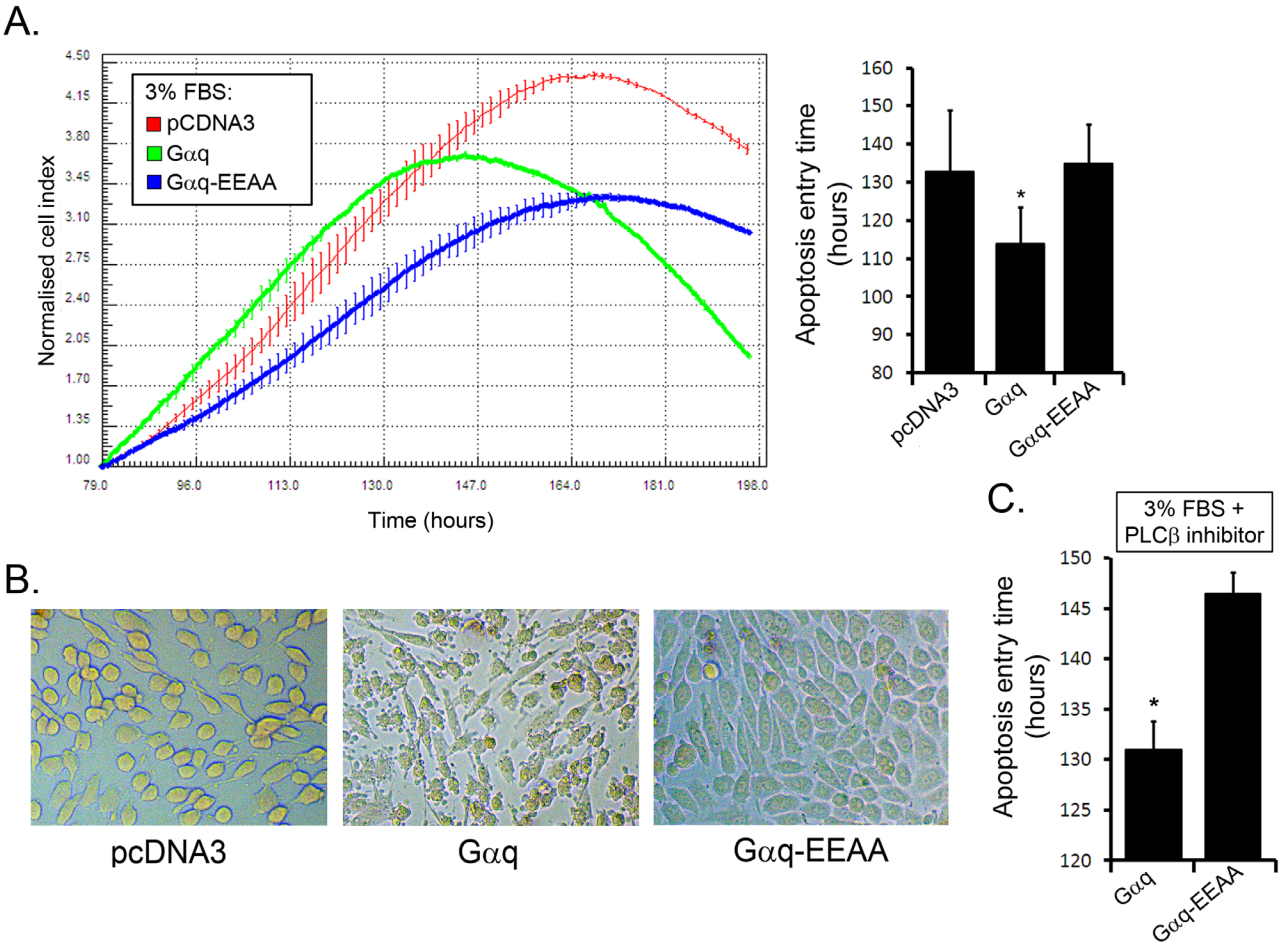

Figure R32. The interaction between Gαq and PKCζ regulates apoptosis entry. Apoptosis entry can be can be readily detected by the xCELLigence software by a characteristic sharp peak in the cell index curve, a rapid switch from positive to negative slope that invariably results in complete cell death. (A) Cho-M3 cells were transiently transfected with GFP and either pcDNA3 empty vector, Gαq wild-type or the Gαq-EEAA mutant. After twenty four hours GFP-positive cells were sorted with a cytometer to guarantee homogeneous cell populations (see Methods) and seeded onto 96-well gold electrode sensor plate in 3% FBS. Cell index changes were monitored every 15min for an average of 140 hours. Apoptosis entry time was determined for each population. Data were the mean +/- SEM of 5 independent experiments (*p<0.05, two tailed T-test). (B) After cytometer sorting, the same cell populations from (A) were seeded onto 48 well plates. Approx. 130 hours after plating, when Gαq but not pcDNA3 or Gαq-EEAA cells had entered apoptosis, they were visualised under a brightfield microscope with a 40x objective. Images are representative of two independent experiments. (C) Cho-M3 cells were transiently transfected with GFP and either Gαq wild-type or Gαq-EEAA, and proceeded as in (A). A PLCβ inhibitor (U73122, 10μM) was added to Gαq and Gαq-EEAA cells before the apoptotic phenotype was detected in any of the cell populations. Apoptosis entry time was determined for each population. Data were the mean +/- SD of 2 independent experiments (*p<0.05, two tailed T-test).

V. DISCUSSION

In the present work, the role of PKCζ as a novel Gαq protein effector has been extensively characterised with particular focus on the biochemical properties of the G protein/effector complex, as well as on the activation mechanisms and cellular repercussions of this new signalling axis. We have determined that the AngII/Gαq/PKCζ/ERK5 pathway occurs in the cardiovascular system and displays Gαq-biased properties. The interaction between the activated Gαq protein, through its pseudo-PB1 type I domain, and its novel effector PKCζ, through its PB1 type II domain, is essential for the activation of ERK5 and is negatively regulated by GRK2. Additionally, the mechanisms of this signalling cascade seem to involve phosphorylation-independent activation of PKCζ and dimerisation. Finally, the Gαq/PKCζ complex is shown to play a role in membrane blebbing, cell growth arrest and apoptosis (graphical abstract in Fig. D1).

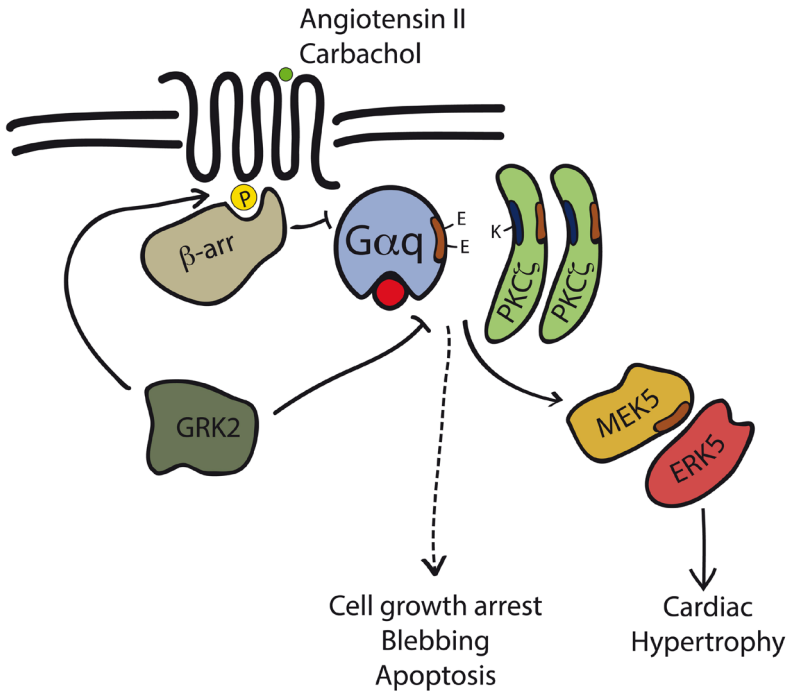

Figure D1. Graphical abstract of the PhD thesis. Integration of data from the different sections of this work in a cartoon model.

The AngII/Gαq/PKCζ/ERK5 pathway in the cardiovascular system

Previously, the occurrence of a novel signalling axis emanating from Gq-coupled GPCRs and mediating the activation of ERK5 had been established in our laboratory (García-Hoz et al., 2010). Briefly, the pathway was described in epithelial cells and involved a direct interaction between Gαq and two novel effectors, PKCζ and MEK5, leading to the activation of ERK5. Neither the activation of PLCβ, the main Gαq effector, nor the transactivation of the EGF receptor, previously shown to activate ERK5 (Kato et al., 1998), were found to be involved in ERK5 activation by Gq-GPCR, thus constituting a non-canonical pathway. Hereby, we have determined that Gαq, a key cardiovascular player involved in hypertrophy and heart failure (Tilley, 2011), initiates the ERK5 pathway in the heart through PKCζ, which might indicate the conservation of the activation mechanism previously described in epithelial cells (García-Hoz et al., 2010). We observe that angiotensin II promotes ERK5 activation in both neonatal and adult murine cardiomyocytes, as well as in neonatal cardiac fibroblasts, although the extent and kinetics of ERK5 stimulation can vary with the cell type or the mice strain. In agreement with our previous findings for other Gq-coupled GPCR in epithelial cells, this process does not appear to involve cross-talk with Gi-coupled signalling, as has been described for angiotensin II-mediated ERK1/2 activation in cardiac fibroblasts (Fielitz et al., 2008). On the other hand, pharmacological inhibition or siRNA-mediated silencing of PKCζ completely abrogated angiotensin-induced ERK5 activation in neonatal and adult cardiomyocytes, or in neonatal cardiac fibroblasts. This is consistent with the lack of activation of this cascade in cardiomyocytes isolated from PKCζ-deficient mice. Despite PKC isoforms such as α, β, ε and ζ expression have been reported to peak in foetal and neonatal hearts, and decrease in adult hearts (Goldberg & Steinberg, 1996), we demonstrate that PKCζ activity is essential for ERK5 activation in adult cardiomyocytes. Overall, these data suggest that PKCζ is required for angiotensin-induced ERK5 stimulation in the two most predominant cell types in the heart. The establishment of a functional pathway in adult cardiac myocytes is relevant for the subsequent description of the pathway in adult murine hearts. As described in the Introduction (section 5d), long-term exposure to angiotensin II showed a strong correlation between the Gαq/PKCζ/ERK5 axis and the development of cardiac hypertrophy (García-Hoz et al., 2012). In this scenario, we were able to determine the activation of the immediate upstream ERK5 MAPKK, MEK5, as well as of the downstream target MEF2c in whole hearts from wild-type but not in PKCζ-deficient mice. Also, we determined the upregulation in wild-type, but not in PKCζ-/- mice, of Ets-1, an important transcription factor whose expression is known to be promoted downstream of ERK5 (Dwivedi et al., 2002). Interestingly, Ets-1 is also a major regulator of Angiotensin-II-induced vascular inflammation and remodelling, thus suggesting that this

role might also be promoted through the Gαq/PKCζ/ERK5 pathway (Zhan et al., 2005). Our data are consistent with previous reports showing that Angiotensin II promotes ERK5 activation in mice myocardium (Ikeda et al., 2005), that cardiac-specific overexpression of either upstream activators (MEK5)(Nicol et al., 2001) or downstream targets (MEF2A and C)(Xu et al., 2006) of ERK5 induces cardiac hypertrophy in mice, and that the activity of ERK5 is increased during left ventricular hypertrophy (Kacimi & Gerdes, 2003; Takeishi et al., 2001), whereas targeted deletion of ERK5 in cardiomyocytes attenuates the hypertrophic response in the heart (Kimura et al., 2010). Overall, our data strongly suggest that the AngII-initiated Gαq pathway leading to ERK5 activation is fully functional in murine hearts and might play an important role in the physiological function of Gαq.

Since we have found that AngII promotes ERK5 activation both in cardiac myocytes and fibroblasts it would be interesting to determine the specific contribution of each cell type in hypertrophy, given the emerging role of cardiac fibroblasts in cardiovascular function and dysfunction (Kakkar & Lee, 2010). This cell type, traditionally linked to the generation of extracellular matrix, has been recently shown to have a very interesting interplay with cardiac myocytes. Indeed, some reports have provided strong evidence for an essential role of cardiac fibroblasts in pressure-overload hypertrophy (Takeda et al., 2010). Moreover, using a myocyte/fibroblast coculture system, it was reported that embryonic cardiac fibroblasts induced proliferation of cardiomyocytes, in contrast to adult cardiac fibroblasts that promoted myocyte hypertrophy (Ieda et al., 2009). This paracrine modulation was found to require the secretion of collagen and fibronectin by fibroblasts and the presence of β1-integrin in cardiomyocytes. Interestingly, it was found that AngII-stimulated wild-type mice had increased cardiac expression of type I α2 pro-collagen and procollagen type IVα, which was not observed in PKCζ-/- mice (García-Hoz et al., 2012). Thus, the possibility that the Gαq/PKCζ/ERK5 axis we have identified in cardiac fibroblasts is positively regulating cardiomyocyte hypertrophy through a paracrine route is an interesting avenue for future research (Fig. D2).

Several subtypes of the PKC family, amongst which PKCζ is found, are known to participate in cardiac fibroblast proliferation. Indeed, pharmacological inhibition of PKCζ was found to decrease basal and TGFβ-induced cardiac fibroblast proliferation (Braun & Mochly-Rosen, 2003). In line with this, preliminary results not included in this thesis suggest a role for PKCζ in Angiotensin-II-mediated cardiac fibroblast proliferation. The role of cardiac fibroblasts in the promotion of fibrosis, characterised by excessive accumulation of extracellular matrix components that correlates with heart dysfunction, is of particular relevance. Indeed, enhanced cardiac fibroblasts proliferation is caused by elevated levels of circulating cytokines and growth factors in events of cardiac hypertrophy and

ischemia, and this is directly linked with fibrosis (Churchill et al., 2008). However, there exists controversy on whether angiotensin exerts direct pro-mitogenic actions in cardiac fibroblasts. Since this hormone is not a strong proliferative factor, its effects are thought to be caused by stimulation of the synthesis of growth or inflammatory substances, like platelet-derived growth factor and cytokines, by integrin activation due to secreted extracellular matrix proteins, or by a combination of these mechanisms (Bouzegrhane & Thibault, 2002). Angiotensin II appears to differentiate cardiac fibroblasts into a growth substance-secreting phenotype, hence it would be difficult to ascertain whether the role of PKCζ is played downstream the angiotensin II receptor or in a secondarily-activated pathway.

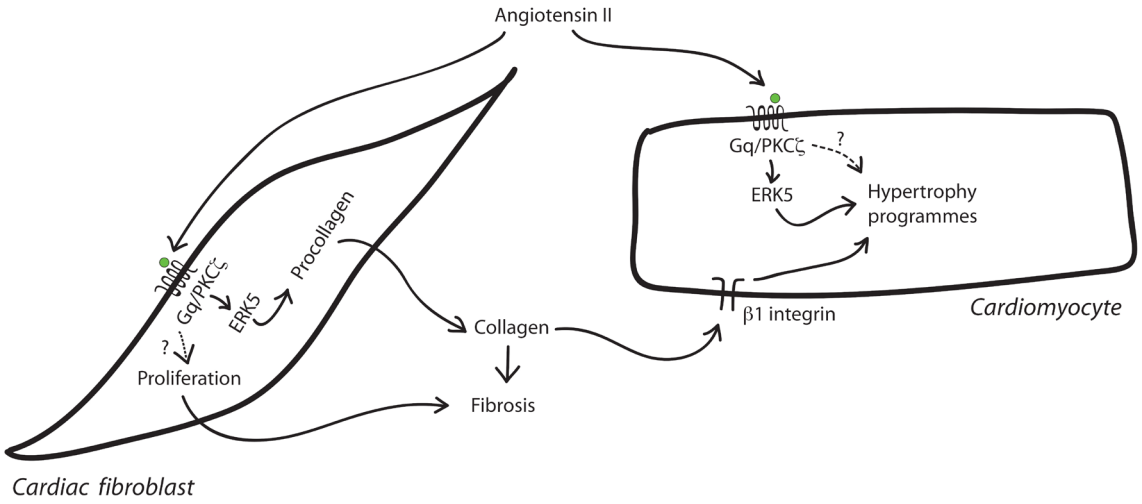

Figure D2. Proposed functions of the ERK5 pathway in the heart. Hypothetic roles of the AngII-activated ERK5 pathway in the interplay between cardiac myocytes and fibroblasts and in the development of cardiac hypertrophy.

In addition to the role of Gαq/PKCζ/ERK5 signalling axis in cardiac myocytes and fibroblasts, the possibility that PKCζ deficiency affects AngII signalling in other cell types, particularly the endothelium (Watanabe et al., 2005), cannot be disregarded. As described in the introduction (sections 3b and 5c) both PKCζ and ERK5 play a key role in endothelial integrity and, in the case of ERK5, this drastically affects heart function during development. Thus, in order to clearly dissect the relative contribution of each relevant cell type in Angiotensin II-mediated hypertrophy, further investigation is required, and would greatly benefit from the use of cell type-specific PKCζ knock-out animals.

In summary, our data point at the Gαq/PKCζ/ERK5 signalling axis as a main player in heart function and disease. Throughout the subsequent sections of the present work, we have sought to identify the biochemical and cellular mechanisms underlying this pathway in an attempt to improve our understanding of its physiological implications.

Gαq-biased properties of the GPCR/ERK5 pathway

The biological functions of GPCR are being currently revised in the light of novel findings on the ability of β-arrestins to promote signalling independently of G proteins. For instance, the activation of ERK1/2 by angiotensin II has been reported as dual; one initial and rapid stage (after 2 minutes) driven by Gαq and a later and more sustained activation (after 10 minutes) driven by arrestins after receptor internalisation (Ahn et al., 2004). Since ERK5 phosphorylation by Gq-coupled GPCR occurs at a relatively late stage after receptor activation (usually peaking around 15/30 minutes) is was tempting to suggest an involvement of β-arrestin through mechanisms dependent on receptor phosphorylation and internalisation. In the same line, the internalization of the p62/PKCζ complex is required for neutrophin-induced ERK5 activation in neurons (Geetha & Wooten, 2003). However, our results clearly show that activation of ERK5 by muscarinic M1/M3 receptors does not require clathrin-mediated or any type of receptor internalisation, which is one of the mechanisms for β-arrestin to initiate signalling. The possibility remained that a β-arrestin-mediated event occurred at the plasma membrane. Indeed, ERK1/2 activation by M3 muscarinic receptor is not affected by receptor internalisation (Budd et al., 1999). However, we found that phosphorylation-deficient muscarinic receptors that cannot recruit β-arrestin or internalise (Kong et al., 2010), managed to activate ERK5 even better than wild-type. Additionally, a β-arrestin-biased ligand failed to activate ERK5 in cardiac fibroblasts. On the contrary, we demonstrate that the overexpression of Gαq greatly potentiates the pathway, as well as an absolute requirement of Gαq for the pathway´s activation through the inability of a binding-deficient Gαq mutant (Gαq-E234/E245-AA) to promote ERK5 stimulation by GPCR (discussed later). To summarise, in all the systems tested, ERK5 activation by GPCR is independent of β-arrestin and displays Gαq-biased properties. Thus, we propose that the unique properties of the Gαq/PKCζ/ERK5 pathway, where Gαq acts as a scaffold (García-Hoz et al., 2010) (a role usually assigned to β-arrestin), could represent a possible common mechanism for Gαq-bias. Indeed, although further investigation is necessary, to our knowledge ERK5 would be the first MAPK pathway not to be activated by β-arrestin. The activation of all the other families of MAPK have been shown to require β-arrestin-signalling to some degree, either through direct interaction between the MAPK and β-arrestin (McDonald et al., 2000), or being activated downstream β-arrestin-initiated pathways (DeWire et al., 2007).

The Gαq-biased properties of the ERK5 pathway open new avenues for the reinterpretation of signalling in heart function and dysfunction. To date, a common approach in heart disease involves inhibitory targeting of GPCR such as β-adrenergic or angiotensin receptors. However, it is widely accepted that there is an urgent need to redesign therapies

in order to minimise secondary effects arising from the fact that both Galpha/β-arrestin-dependent pathways are inhibited by this unbiased approach. It seems that receptors have evolved to initiate cardio-protective pathways through β-arrestins that would compensate for the cardio-damaging Gαq signalling (Noma et al., 2007). Since a dual blockage of these antagonistic effects is not beneficial, a new class of specifically biased drugs is starting to be developed with promising results (Violin et al., 2010). In addition to this, we propose that the Gαq-biased ERK5 pathway would have strictly deleterious effects, as recently demonstrated in cardiac hypertrophy (García-Hoz et al., 2012), and would make a potential target for therapeutics.

The Gαq/ PKCζ complex

The primary protein complex formed in the Gαq-ERK5 pathway involves the activated Gαq protein and its effector PKCζ. It has previously been described that Gαq interacts directly and in a stimulus-dependent manner with PKCζ and this is the earliest event that correlates with the activation of the ERK5 cascade (García-Hoz et al., 2010). However, no causal relationship had yet been established between the Gαq/PKC complex and the activation of ERK5.

As a first approach to determine the biological significance of the PKCζ/Gαq complex we studied the association of these two proteins in living cells. The occurrence of protein complexes in a cellular scenario is a strict requirement for its functionality and can be detected through novel techniques such as the protein complementation assay (PCA) (Michnick et al., 2011). We utilised the Venus-YFP PCA due to the feasibility to irreversibly trap transient protein complexes. Indeed, the formation of a high-affinity complex between Gαq and PKCζ was readily detected. The complex was selective since Gαq did not interact with another PKC family member, PLCβ, and PKCζ did not interact with another Galpha family member, Gαi1. These data are consistent with our coimmunoprecipitation experiments that previously showed the family-specificity of the Gαq/PKCζ association (García-Hoz et al., 2010). The localisation of the protein complex was invariably cytoplasmic which poses questions on whether this is a reliable representation of reality, since Galpha subunits are predominantly localised at the plasma membrane. Tagging of G alpha proteins at either the N or the C terminus may affect localisation and effective coupling to effectors and receptors. However, a PCA-based approach recently tagged Gαi1 and successfully assayed its interaction with PKA regulatory subunit at the plasma membrane (Stefan et al., 2011). Thus, the question remains of whether the PKCζ/Gαq complex is mislocalised due to tagging of the G protein or it actually resides in the cytoplasm. One piece of evidence that supports this latter possibility refers to the dynamics of the complex

upon ligand addition. We have observed that fluorescent signal transiently concentrates towards cell membrane protrusions (blebs) upon carbachol stimulation (see below). Since YFP reconstitution is an irreversible event, these data suggest that, either new protein complexes are being formed in these cellular blebs or already preformed complexes are being redistributed towards these localisations. The possibility of simple diffusion as a cause for the redistribution is disregarded since this event would only explain an even distribution of complexes throughout the cell, but not a marked concentration of fluorescence at specific localisations, as it was observed. In sum, our data indicate that the PKCζ/Gαq protein complex is selectively formed in living cells, although its precise subcellular localisation awaits further investigation. The ongoing generation of internally-tagged versions of G alpha subunits with renilla luciferase for utilising in bioluminescence resonance energy transfer (BRET) assays (Dr. Michel Bouvier, personal communication) is expected to shed light on the localisation of the Gαq/PKCζ complex.

Regarding the specificity of the complex, it is of particular importance the surprising fact that PKCζ does not interact with Gα11 as detected by both immunoprecipitation experiments and the *in vivo* PCA assay. Gαq and Gα11, the only ubiquitously expressed members of the Gαq/11 family, are highly homologous proteins (90% aminoacid identity) and have been considered to play complementary roles, and to indistinctively activate the same cellular effectors (Hubbard & Hepler, 2006). Our results indicate that PKCζ is likely the first differential effector within the Gαq/11 family. This finding poses new exciting questions regarding the formation of the Gαq/PKCζ complex (see below) and the differential functions of Gαq and Gα11.

There is a relative paucity of reported differential functions between the main members of the Gαq/11 family. An assumed functional redundancy has lead to a generalised use of experimental tools that do not differentiate between these two proteins (e.g. anti-Gαq/11 antibodies). Indeed, only double Gαq/Gα11 knockout mice models show drastic cardiac abnormalities whereas mice with single deletions are seemingly normal. However, there are some differential roles in which only Gαq, but not Gα11, is involved. These refer to certain platelet functions, since only Gαq is expressed in platelets and specific Gαq KO mice show longer bleeding times, as well as to craniofacial defects that are also specifically shown by Gαq-/- mice (Hubbard & Hepler, 2006). Additionally, it has been shown that Gαq, but not Gα11, is involved in glutamate receptor-dependent long-term depression in the hippocampus (Kleppisch et al., 2001). Also, it is interesting to note that diabetic Gα11 knockout animals show a decreased expression of PKCζ in coronary arteries, thus suggesting that this might be a compensating effect for an enhanced Gαq signalling through PKCζ (Hoyer et al., 2010). Addressing whether PKCζ, as a selective Gαq effector, accounts for

some of the functional differences between Gαq and Gα11 remains an interesting research avenue.

The characterization of the PKCζ/Gαq complex required an insight into the particular surfaces involved in complex formation. Initial experiments indicated the strict requirement of PKCζ PB1 domain for ERK5 activation by Gq-coupled GPCR and for Gαq interaction. This domain has previously been involved in a number of protein-protein interactions required for processes such as NFκB activation or cell polarity (See Fig I9). Since PKCζ harbours two types of PB1 domains (type I and type II), we studied the type II domain (with basic properties), which has been involved in ERK5 activation by other stimuli (Diaz-Meco & Moscat, 2001). We found that lysine in position 19 (K19), a main driver of PB1-PB1 interactions (Hirano et al., 2005), was a crucial aminoacid for PKCζ to interact with Gαq. Interestingly, it has been previously reported that Gαi associates with the PB1-type II of p67phox and this results in an enhanced activity of the G protein (Marty et al., 2006). With regards to the PKCζ binding region in Gαq we have identified a pseudo-PB1 domain within the Gαq effector domain that shows a striking similarity with MEK5 PB1 type I domain, suggesting that this could be a conserved PKCζ-binding region. Indeed, we demonstrated that mutations on two residues of this domain, E234/E245, abrogated PKCζ binding. The positions of aminoacids E234 and E245 in Gαq are highly conserved in PB1 proteins such as MEK5 or p62 and coincide with crucial clusters (A1 and A2) for the function of these PB1 domains (Hirano et al., 2005). It is thus tempting to suggest that electrostatic interactions between positively charged lysines (such as K19) and arginines in the PKCζ PB1-type II domain, and glutamic acids and aspartic residues in Gαq are key determinants for the formation of the complex. It should be noted that the involved Gαq region was termed pseudo-PB1 domain, since the three-dimensional fold is not that of canonical PB1 domains despite striking similarities in the primary sequence. Our data suggest, however, that the presence of some key charged residues would allow specific interactions between classical and pseudo PB1 domains. In fact, preliminary data indicate that Gαq is indeed able to bind other PB1 proteins through its pseudo-PB1-type I domain (data not shown in this thesis).

The position of the PKCζ-binding region in Gαq sheds light on several aspects of the complex. First, the fact that the pseudo-PB1 domain lies within the effector binding area supports the notion that PKCζ is a *bona-fide* effector of Gαq. It was previously shown that G alpha protein effectors invariably associate to the effector binding region but the subsets of crucial aminoacids for these associations vary with the specific effector (Oldham & Hamm, 2006). Hence, we demonstrate that PKCζ binds a different subset of aminoacids to those previously described for other Gαq effectors (PLCβ, p63RhoGEFs, GRK2).

Consistently, we find that GRK2-binding impairing mutations in Gαq did not affect PKCζ association nor did PLCβ-binding impairing mutations interfere with ERK5 activation (García-Hoz et al., 2010).

In this context, our finding that Gα11 does not interact with PKCζ poses some intriguing questions. An analysis of the sequence alignment between Gαq and Gα11 (Appendix, Fig. 1A) shows a high overall homology (90%) that reaches 100% in the C-terminal end, so the effector binding region, identified to bind PKCζ, is completely conserved in both proteins. The position of variable clusters II and III (cluster I is disordered and has not been crystallised in any of the Gαq structures) in the three-dimensional structure is very distant from the PKCζ binding site (Appendix I, Fig. 1B); hence it is unlikely that these residues would provide contacts for PKCζ. It would still be possible that the disordered N-terminus (Cluster I) would be more proximal to PKCζ than the other clusters. However, it may well be that the absence of interaction between Gα11 and PKCζ is not due to changes in specific aminoacids responsible for PKCζ binding. Instead, we propose that this phenomenon is a matter of pre-steady state kinetics: a differential availability of protein partners before the binding reaction that leads to differential association, being Gαq and PKCζ more prone to interact due to a similar subcellular localisation. In this line, cluster I at the N-terminus, where some of the divergent aminoacids between Gαq and Gα11 are found, is known to be crucial for membrane localisation. This predominantly basic region interacts with membrane lipids and also contains palmitoylated residues that target the G proteins to specialised membrane microdomains like lipid rafts (Hubbard & Hepler, 2006). There are reports for agonist-dependent localisation of both Gαq (Dave et al., 2009) and PKCζ (Hajduch et al., 2008) in lipid rafts enriched in caveolae. Particularly, Gαq is often used as an experimental marker of lipid rafts (Qiu et al., 2011). Thus, it would be interesting to explore whether lipid rafts, or other membrane microdomains, would provide specific microenvironments for the formation of the Gαq/PKCζ complex.

In sum, although we have roughly identified the binding regions for the PKCζ/Gαq complexes through mutagenesis studies, no claims can be made regarding the precise molecular architecture of the complex, and further structural work needs to be carried out to specifically characterise the surfaces involved. In this line, we are currently performing molecular dynamics and protein docking simulations to determine other potential binding sites of PKCζ in the threedimensional structure of Gαq (ongoing collaboration with J. Klett). The mutants generated for Gαq are, however, very useful tools for studying the cellular events that depend upon the formation of a Gαq/PKCζ complex. Indeed, we demonstrate the strict requirement of ERK5 activation by Gq-GPCR on the Gαq/PKCζ complex. A constitutively active mutant Gαq harbouring the E234/E245-AA mutation was unable to

activate ERK5, and similarly, the same mutation on a wild-type background impaired Gαq enhancement of ERK5 activation by GPCR. In parallel experiments, expression of the Gαq E234/E245-AA mutant still activated inositol phosphate signalling (although to a lesser extent than wild-type Gαq) and did not impair the full stimulation of ERK1/2, that lays downstream of DAG-activated PKC. Overall, these data constitute the first direct demonstration of a biological function for the protein complex between novel effector PKCζ and Gαq, and supported the reliability of the mutagenesis strategy.

Effect of Gαq modulators on Gαq/PKCζ complex formation and dowstream signalling

We were also interested in addressing the modulation of the Gαq/PKCζ complex and the resulting effects on the activation of the ERK5 cascade. In the case of Gαq signalling, the two main modulators are GRK2 and RGS2/4 proteins. GRK2 phosphorylates and desensitises activated GPCRs and also negatively regulates Gαq activity through a sequestering effect. This has been described as a strong competition between the kinase and effectors, that displaces the latter from the Gαq binding site and dampens downstream signalling (Carman et al., 1999). Gαq can also be deactivated by RGS proteins, which enhance the GAP activity of Gαq to promote GTP hydrolysis. The latter proteins have been reported not to compete with the effector binding site. In agreement with this notion, we demonstrate that Gαq/PKCζ complexes are undisturbed by the overexpression of RGS2 and 4. Similar results have been reported for other known Gαq partners that associate with the effector binding site, such as p63rhoGEF and GRK2 (Shankaranarayanan et al., 2008). This strengthens the idea that PKCζ is a bona-fide effector and opens the possibility to the formation of a ternary complex whereby RGS allosterically, but not sterically, modulates PKCζ binding to Gαq.

On the other hand, we report that GRK2 sterically impedes the association of PKCζ to Gαq *in vitro* and in living cells, and abrogates ERK5 activation due to Gαq sequestering and to receptor desensitisation. GRK2 was also able to down-regulate the association of Gαq to MEK5, the other Gαq effector in the ERK5 pathway, which suggests that MEK5-Gαq association also requires the effector-binding surface of the G protein. Additionally, we show that the impairment of the GRK2/Gαq complex with a specific association-deficient Gαq mutants upregulates the Gαq/PKCζ complex and greatly enhances ERK5 activation. This suggests that cellular contexts in which GRK2 levels are decreased would allow for hyperactive signalling of the ERK5 pathway. Inversely, the fact that GRK2 overexpression downregulates the Gαq/PKCζ complex and ERK5 activation also suggests that similar events will be occurring in cellular contexts with increased GRK2 levels. Indeed, GRK2 expression or functionality is altered in a number of physiopathological conditions where,

according to our findings, Gαq signalling towards the ERK5 pathway might be modified. In the cardiovascular system GRK2 is known to play a key role (see Introduction, section 6d) through the desensitisation of β-adrenergic receptors and resulting modulation of cardiac contractility (Penela et al., 2010). In failing human hearts increased GRK2 levels have been associated to a decrease in β-adrenergic signalling (Ungerer et al., 1994). Additional pathways would similarly be downregulated in this context which poses for questions regarding the triggering event of cardiac failure. Our experiments in the PKCζ-/- mouse model only address the role of the Gαq/PKCζ/ERK5 axis in heart hypertrophy and have not investigated the potential role in heart failure (García-Hoz et al., 2012). However, it is tempting to speculate that increased GRK2 levels would lead to an inhibition of the ERK5 pathway in the transition from hypertrophy to heart failure. The overexpression of GRK2 in the myocardium also attenuates angiotensin II-mediated increases in contractility and heart rate (Rockman et al., 1996). Additionally, inhibitors against MEK1/2 and MEK5 have been shown to reduce contractility increases promoted by the endothelin-1 receptor, another prevalent Gq-coupled receptor in the heart (Münzel et al., 2005). Thus, we suggest that GRK2 might regulate the contractile effects of Gq-coupled angiotensin and endothelin receptors through the inhibition of the ERK5 pathway. In conclusion, the functional relationship between GRK2 and the Gαq/PKCζ/ERK5 pathway in different pathological settings deserves to be addressed in future research projects.

Activation mechanisms of the Gαq/PKCζ/ERK5 pathway

Once established that the interaction between Gαq and PKCζ is necessary for the stimulation of the ERK5 pathway by GPCRs it remained unclear how this interaction triggered the activation of the cascade. Our starting assumption was that the kinase activity of PKCζ was involved since the PKCζ-pseudosubstrate catalytic inhibitor abrogates ERK5 activation by Gq-GPCR in a number of systems [(García-Hoz et al., 2010), (García-Hoz et al., 2012) and results within this thesis]. Several mechanisms have been described for atypical PKC activation (see Introduction section 3b). Insulin provokes increases in PKCζ enzymatic activity through PDK-1-dependent T410 phosphorylation in the activation loop, T560 autophosphorylation at the turn motif, and phosphorylation-independent/conformational-dependent relief of pseudosubstrate autoinhibition (Standaert et al., 2001). However, we did not found changes in the phosphorylation status of either T410 or T560 in response to Gq-GPCR stimulation. Still, we could detect basal phosphorylation on both residues consistent with other reports that suggested that most of the Thr410 of PKCζ is phosphorylated following ectopic expression in HEK-293 cells (Smith & Smith, 2002). Since it is uncertain whether PKCζ is catalytically active if not phosphorylated on Thr410 (Smith & Smith, 2002), the pathway might require a previously phosphorylated

form of the kinase. Regardless, it is increasingly clear that additional activation mechanisms can operate on PKCζ independently of phosphorylation, which is evidenced by the ability of insulin to activate the phospho-mimetic mutant PKCζ-T410E/T560E (Standaert et al., 2001). Thus, we postulate that a conformational-dependent relief of pseudosubstrate autoinhibition could be operating between Gαq and PKCζ. Consistent with this notion, the activation mechanism exerted by Gαq on main effectors, PLCβ and p63RhoGEF, involves the allosteric relief of an autoinhibitory loop buried within an active region promoted upon Gαq binding (Shankaranarayanan et al., 2010; Waldo et al., 2010). A similar mechanism involving the disruption of an intramolecular inhibitory interaction in the effector has been proposed for Gαq-mediated Btk activation (Ma & Huang, 1998). Regarding PKCζ, it has been recently described that PB1-PB1 interactions with Par6 allosterically activate the kinase by pseudosubstrate displacement (Graybill et al., 2012). Since we have shown that Gαq interacts through a pseudo-PB1 domain with the PB1 type II domain PKCζ, we suggest that a similar activation mechanism might be taking place between Gαq and PKCζ (Fig. D3).

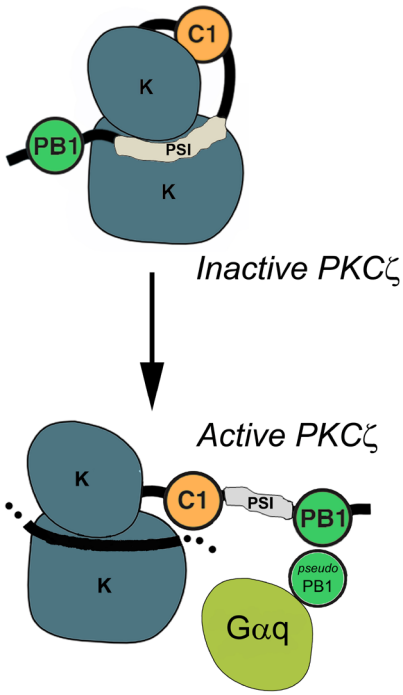

Figure D3. Proposed activation mechanism of PKCζ by Gαq. Gαq might activate PKCζ in a phosphorylation-independent manner through a PB1-pseudoPB1 interaction that relieves autoinhibition of the kinase domain by the pseudosubstrate region.

As described throughout this thesis, protein molecules form modular complexes that are required for correct signal propagation. Protein dimerisation is a common event in the function of numerous enzymes, ion channels, receptors or transcription factors. This process of self-association has been positively selected throughout evolution as it minimises genome size and still allows for modular organisation (Marianayagam et al., 2004). Particularly, PB1 domains are known modules for homo/hetero- dimer/oligomer

formation (Noda et al., 2003). Proteins harbouring two types (I, II) of PB1 domains such as p62 are known to oligomerise in a process where the basic cluster of one p62 molecule binds to the acidic motif of another in a front-to-back arrangement (Wilson et al., 2003). Since PKCζ also harbours two PB1 domains, we investigated whether dimerisation could be an additional event downstream GPCR activation, along with the formation of heterodimers between PB1 domains from MEK5 and PKCζ, or between pseudo-PB1 and PB1 domains of Gαq and PKCζ. Thus, we used the protein complementation assay with PKCζ tagged to different fragments of YFP and we could detect high-affinity dimerisation in living cells. Most interestingly, by using a reversible PCA with renilla luciferase fragments, we determined that PKCζ dimerisation is transiently induced by Gq-coupled GPCR stimulation *in vivo*. PKCζ dimerisation occurs in a coincident time-course (between 2 and 10min) to Gαq-PKCζ interaction after receptor stimulation (García-Hoz et al., 2010), which suggests that dimerisation might be necessary for PKCζ function in Gαq signalling. Also, our results clearly indicate that there is a transient increase of PKCζ molecules in a particular cellular location driven by GPCR stimulation. Since PKCζ is known to directly associate with Gαq, MEK5 and ERK5, there is a possible scenario in which PKCζ could oligomerise and serve as a signalling platform for the activation of ERK5. An analysis of the PLCλ-Par6α crystal structure, shows that the PB1-type I domain of PLCλ is free to interact with other PB1-harbouring proteins (Appendix I, Fig. 2). Since PKCζ and PLCλ PB1 domains are structurally very similar, we suggest that the spatial disposition of PB1 domains I and II in PKCζ would allow for oligomerisation of several molecules in a front-to-back arrangement, as previously described for p62 (Wilson et al., 2003). Additionally, since it is known that PB1-PB1 interactions can result in PKCζ activation, it is tempting to suggest that activation events might occur as a result of PKCζ oligo/dimerisation. Thus, we propose that PB1-driven PKCζ dimerisation might be a cellular mechanism for imposing spatial and temporal specificity during Gαq-initiated signalling.

Also relevant to our understanding of protein complex architecture in the Gαq-ERK5 pathway, we have found that PKCζ interacts with ERK5 in a stimulus-dependent manner, at a coincident time-frame with dimerisation and Gαq/PKCζ complex formation. In addition, we demonstrated that Gαq and ERK5 are found together in an activated macromolecular complex through an interaction with PKCζ. These results are consistent with the scaffold role of PKCζ in the ERK5 cascade.

Based on all the information collected (including binding regions and activation and deactivation mechanisms), we propose a global working model to account for the sequential events and macromolecular complexes involved in ERK5 activation by Gq-coupled GPCR (Fig. D4): Ligand binding to the receptor causes Gαq activation (1) which, in turn, promotes an interaction with PKCζ (2). The Gαq/PKCζ complex is formed between the PB1 domain type II of PKCζ (shown in blue) and the pseudo-PB1 type I of Gαq (shown in brown). We hypothesise that Gαq would then allosterically activate PKCζ by relieving autoinhibition exerted by the pseudosubstrate domain (2). Open conformation of the kinase due to activation and high concentration of PKCζ molecules in specific Gαq-directed cellular location favours the formation of dimers/oligomers of PKCζ (3). Additional activation events might occur in the dimerisation process between different PKCζ molecules due to PB1-PB1 interactions and pseudosubstrate relief. In an open (active) conformation, the kinase domain of PKCζ is free to interact with ERK5 [as previously described in (Nigro et al., 2010)], which is recruited by PKCζ into a multimolecular complex together with Gαq (4). Next, MEK5 is recruited into the signalling complex through a direct interaction with activated Gαq that does not involve MEK5 PB1 domain (García-Hoz et al., 2010) (5). Eventually, a transient complex between MEK5, Gαq and PKCζ/ERK5 is formed. Gαq acts as an adaptor bringing PKCζ and MEK5 into close proximity (García-Hoz et al., 2010). This intermediate complex rapidly progresses to MEK5 binding PKCζ with high affinity and Gαq displacement from the PB1 domain type II of PKCζ (García-Hoz et al., 2010), so Gαq leaves the multimolecular complex (6). The formation of PKCζ-MEK5 complexes might contribute to decreasing PKCζ dimers due to PB1-PB1 competition between different PKCζ molecules and MEK5. From this point onwards we speculate that the activation mechanisms involved are similar to those reported for EGF-mediated ERK5 activation (Diaz-Meco & Moscat, 2001). Subsequently, the interaction between MEK5 and PKCζ favours MEK5 autophosphorylation (7), and PKCζ acts as scaffold in the activation of the MAPK cascade by allowing MEK5 to phosphorylate ERK5 (8). Activated ERK5 will be able to phosphorylate several substrates and initiate diverse cellular functions. GRK2 and RGS proteins would act as negative modulators of this cascade by sequestering Gαq away from PKCζ (2') or from MEK5, or by binding to Gαq in the complex to promote GTPase activity and deactivation of Gαq (3´), respectively. This model may serve as a theoretical framework for the subsequent studies on the pathway.

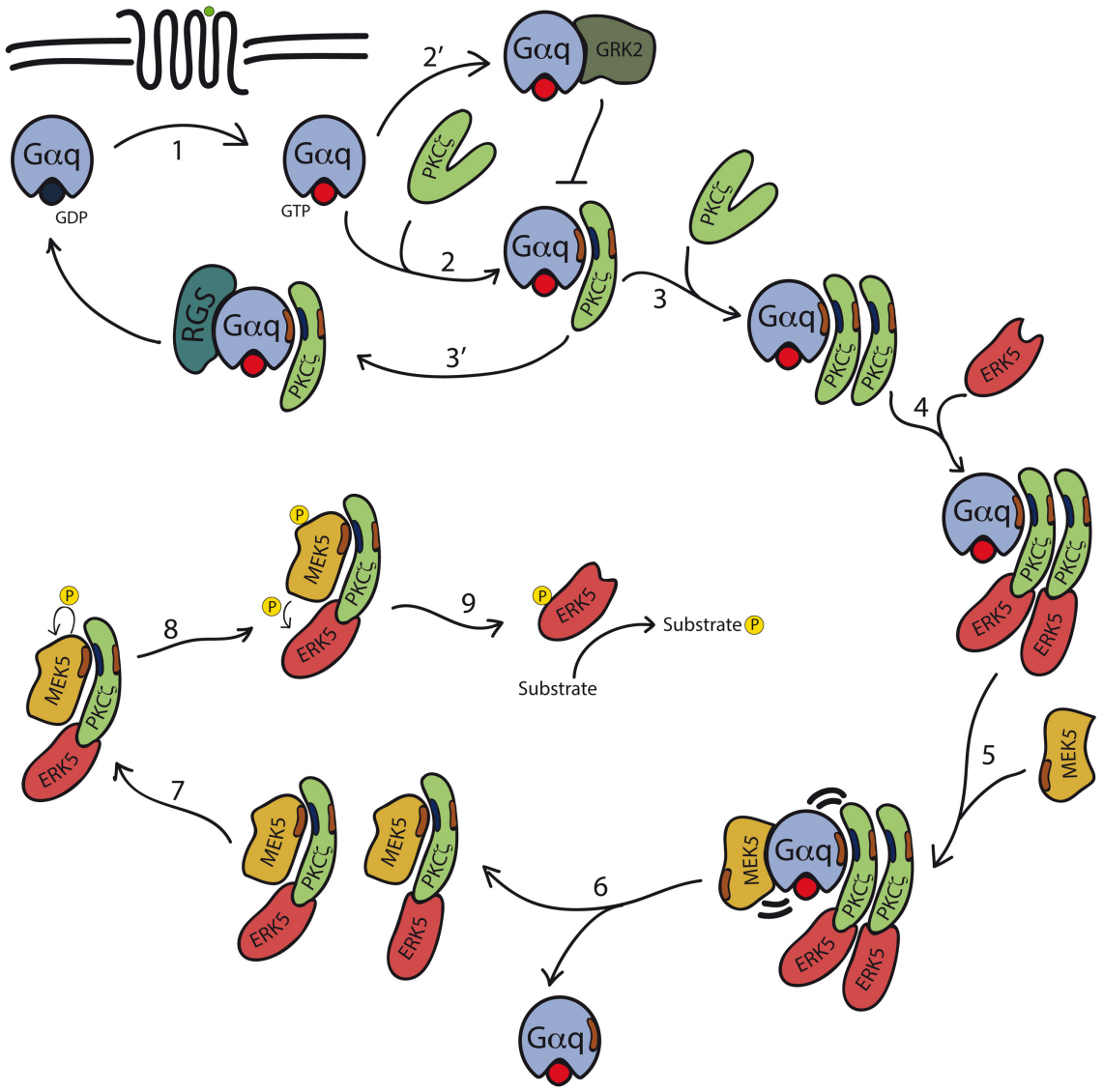

Figure D4. Model of the activation of ERK5 by Gq-coupled GPCR. Proposed sequential formation of protein complexes involved in the Gαq-ERK5 pathway. PB1 type I domain and pseudo-PB1 type I domain are shown in brown, PB1 type II in blue. See text for detailed information.

A number of questions remain to be addressed. For instance, the interplay between PKCζ and ERK5 activities has to be clarified. On the one hand, we have shown that the presence and a pseudosubstrate-inhibited function of PKCζ is strictly required for the phosphorylation of ERK5 in response to Gq-coupled GPCR stimulation (muscarinic M1 (García-Hoz et al., 2010) and M3, and angiotensin II (García-Hoz et al., 2012) receptors), whereas it was previously reported that PKCζ protein, but not its kinase activity, was required for ERK5 phosphorylation upon EGFR stimulation (Diaz-Meco & Moscat, 2001). On the other hand, in our gene expression studies performed in AngII-treated mice we showed that both ERK5 and several products downstream from ERK5 were upregulated in wild-type

animals compared to PKCζ-/- mice (ETS-1, NPPA, Col1α2, BCL2, CCNA2)(García-Hoz et al., 2012), consistent with the notion that ERK5 transcriptional activity is enhanced by PKCζ. However, a recent publication reported that TNF-α promoted ERK5-PKCζ association in the endothelium which resulted in an ERK5 phosphorylation in Ser486 that inhibited its transcriptional activity (Nigro et al., 2010). Although the phosphorylation was not endogenously detected, the authors showed that the sole expression of PKCζ or its catalytic domain was sufficient to decrease ERK5 transcription of a luciferase reporter gene. These seemingly contradictory findings might provide the basis for a novel regulation mechanism of ERK5 activity through GPCR. In this model PKCζ would be required for the activatory phosphorylation of ERK5 in its kinase domain (TEY motif) and additionally PKCζ would itself phosphorylate the C-terminus of ERK5 (Ser486) thus inhibiting its transcriptional activity. It is still unclear whether this mechanism would only operate in the endothelium, since it does not fit with our results in whole hearts (containing cardiomyocytes, cardiac fibroblasts and endothelial cells). Thus, the interplay between PKCζ and ERK5 could be more complex than expected and deserves a detailed characterization in each particular scenario.

The Gαq/PKCζ axis is involved in membrane blebbing, cell growth arrest and in the promotion of apoptosis

In an attempt to expand our understanding of the functionality of the Gαq/PKCζ complex, the last section of the present work was devoted to characterising the participation of this protein complex in diverse cellular effects, using the XCELLigence system (Roche) due to its unique features for real-time monitoring of cell proliferation, cell surface coverage, cellular adhesion strength or cell viability (see Methods).

Acetylcholine muscarinic receptors are involved in the regulation of a wide variety of physiological responses, including apoptosis, cellular proliferation and neuronal differentiation (Caulfield, 1993). Indeed, muscarinic signalling has been shown to be abnormal in many diseases, such as overactive bladder, chronic obstructive pulmonary disease, neurodegenerative diseases, Sjogren's disease, vascular dementia and others (Resende & Adhikari, 2009). The stimulation of the Gq-coupled muscarinic M1 and M3 receptors by carbachol results in measurable phenotypical changes; the most immediately noticeable effect is plasma membrane blebbing (Street et al., 2006). Blebs are spherical protrusions of the plasma membrane that result from actinomyosin contractions of the cortex (Charras, 2008). These processes in which the cytosol steams out of the cell body, are reported to have a transient nature lasting for approximately 40min-1hr, after which time cells retract to their basal state (Meshki et al., 2009). Bleb expansion does not involve actin polymerisation, which distinguishes blebs from all other known cellular protrusions, but

instead they are a result of local disruption of membrane-actin cortex interactions. Blebbing correlates with changes in electrical impedance measured in cellular monolayers with the XCELLingence software (Meshki et al., 2009). In Cho-M3 cells, blebbing was detected by a sharp increase in the cell index indicative of a higher cell surface coverage that peaked after approximately one hour of ligand addition. This effect showed a clear dose-dependence on carbachol concentration. Interestingly, cells overexpressing wild-type Gαq displayed a greatly enhanced carbachol-promoted blebbing whereas those expressing the Gαq-EEAA (E234/E245-AA) mutant behaved as control, empty vector-transfected cells. Moreover, we find the Gαq/PKCζ complex localised in blebbing processes upon carbachol stimulation. These data suggest that PKCζ interaction with Gαq is essential for M3 receptor-induced blebbing processes upon carbachol addition, supporting the notion that PKCζ is a functional Gαq effector that plays relevant roles in different cellular functions.

Classically, muscarinic-induced reorganisation of both actin and myosin has been tied to the activation of RhoA (Linseman et al., 2000; Strassheim et al., 1999) and that of its main downstream target Rho associated kinase (ROCK)(Chernyavsky et al., 2004). Interestingly, the angiotensin II type I receptor (AT1R) was shown to induce blebbing in a process that involved RhoA, ROCK and myosin light chain kinase but was independent of PLCβ activation (Godin & Ferguson, 2010). Contradictory evidence was shown by a recent report in which the muscarinic M1 receptor induced blebbing through reversible remodelling of the spectrin cytoskeleton in Cho cells (Street et al., 2006). The authors showed that this effect was dependent on PLCβ, conventional PKC and ROCK, but independent of RhoA activation. Thus, it seems that different pathways can promote blebbing through different mechanisms downstream Gq-coupled GPCR. Our results suggest that the M3 receptor-induced blebbing in Cho cells requires the association between Gαq and PKCζ but the particulars of this signalling axis have not yet been determined. It is unclear whether the ERK5 pathway is located downstream of the PKCζ/Gαq complex in carbachol-induced blebbing or, alternatively, other pathways are emanating from this functional relationship. ERK5 has been shown to promote actin cytoskeleton disruption in Src-transformed cells, which was required for an oncogenic phenotype (Barros & Marshall, 2005). Since actin disruption is a hallmark of blebbing, we suggest that ERK5 activation downstream the Gαq/PKCζ complex might provide a feasible explanation of the most distinctive feature of blebbing. Additionally, PKCζ has been shown to associate with actin upon insulin stimulation of Cho cells and to participate in the remodelling of the actin cytoskeleton (Liu et al., 2007). Since Gαq has also been shown to associate to actin (Mizuno & Itoh, 2009), the Gαq/PKCζ complex might form in actin-rich regions and thus promote ERK5 activation that would lead to cytoskeleton disruption. Alternatively, PKCζ has been reported to activate RhoA downstream of the Gq-coupled thrombin receptor in the endothelium and

to initiate a pathway involving ROCK and myosin light chain kinase that leads to increased endothelial permeability (Minshall et al., 2010). Interestingly, the authors suggest that there might be a link between Gαq and PKCζ that had not yet been described. Thus, we speculate that the Gαq/PKCζ activated complex might have a role in promoting RhoA activation independently of the Gαq effector p63RhoGEF. In summary, we propose two possible mechanisms to explain the observed abrogation of muscarinic M3 receptor-induced blebbing upon Gαq/PKCζ complex disruption: ERK5-mediated actin cytoskeleton disruption or the activation of RhoA downstream the Gαq/PKCζ complex (Fig. D5).

To date, the function of blebbing is far from being understood. Blebs are known to contribute to cell movement (Fackler & Grosse, 2008), as described for other membrane protrusions like lamellipodia or filopodia, but in other contexts such as cytokinesis and apoptosis, blebbing function is much speculative (Charras, 2008). As for receptor-induced blebbing, though being a known cellular effect, its function is unknown. Some authors have suggested a role for blebs in providing a dynamic and reversible signalling platform to the specific domains of the plasma membrane in response to stimulation of GPCR (Street et al., 2006). Since the visualisation of the Gαq/PKCζ complex in living cells showed a clear concentration at these protrusions (discussed above), this opens the possibility for selective localisation of Gαq/PKCζ signalling at cellular blebs. Thus, we speculate that blebs require the formation of a Gαq/PKCζ complex and might additionally provide a specific signalling microenvironment for this signalling axis.

Generally speaking, muscarinic receptors were initially thought to mediate cell survival and antiapoptotic responses (Gutkind et al., 1991). However, in the Cho-M3 cell line, used throughout this thesis, stimulation of the muscarinic M3 receptor leads to inhibition of cell growth and apoptosis. Carbachol-mediated stimulation of these cells resulted in cell growth arrest at the G1 phase that correlates with JNK activation and p21cip1/waf1 increased expression (Burdon et al., 2002). Opposite results were obtained for the stimulation of the Cho-M2 cell line, suggesting that the effect observed is dependent on Gαq/11-coupling of the receptor. Indeed, the activation of Gq-coupled muscarinic receptors (M1 and M3) has been shown to inhibit cell proliferation in a number of cell lines (Shafer & Williams, 2004). In our experiments, we observed a clear dose-dependent cell growth arrest effect of carbachol in Cho-M3 cells that was reliably detected through the XCELLigence system. This effect, which was also detected in the 3T3-M1 cell line (data not shown), is transient and, invariably, cells started growing again after approximately 72 hours of ligand addition. The analysis of this process in cells overexpressing either Gαq wild type or Gαq-EEAA (E234/E245-AA) showed that Gαq cells had a more drastic decrease of cell proliferation upon carbachol addition than control (pcDNA cells) or Gαq-EEAA cells, both of which behaved indistinctively. This suggests that an absence of interaction between

Gαq and PKCζ abrogates the cell growth arrest effect of carbachol-activated Gαq. The specific effects downstream of the Gαq/PKCζ complex that contribute to cell growth arrest are unknown. ERK5 effects have been virtually always associated to the promotion of cell proliferation as it activates NFκB and promotes the expression of cyclin D1, both of which contribute to cell cycle progression (Cude et al., 2007; Mulloy et al., 2003). The effects reported for carbachol-induced cell growth arrest involve the inhibition of CDK2, cyclin A and cyclin D, and the arrest at the S phase (Burdon et al., 2002). Thus, ERK5 reported roles do not seem to fit with our results and it is likely that other players are acting downstream the Gαq/PKCζ complex in this process. Since most anti-proliferative actions of carbachol in Cho-M3 cells have been tied to JNK activation, it would be interesting to address whether the interaction between Gαq and PKCζ affects this pathway. Tiam1, a known upstream activator of Rac in the Gq-GPCR-initiated JNK cascade (Buchsbaum et al., 2002), has been shown to form an active complex with Par proteins and PKCζ that controls polarity in keratinocytes (Pegtel et al., 2007). Thus, it is tempting to speculate with the possibility that a similar complex would be promoted by the Gαq/PKCζ signalling axis leading to JNK activation. Indeed, several studies have found PLCβ-independent activation of JNK. Muscarinic M1 receptors in NIH-3T3 cells promoted JNK activation at a different timeframe from other MAPK pathways and entirely independently of DAG-activated PKC (Coso et al., 1995). Also, the activation of JNK downstream the α1A-adrenergic receptor was found to be both calcium- and PKC-independent in PC-12 cells (Berts et al., 1999). Therefore, the activation of Tiam1 by the Gαq/PKCζ complex would not only provide a feasible explanation for its participation in cell growth arrest (Fig. D5), but also a novel mechanism for the Gαq-mediated activation of JNK independently of PLCβ.

Other reports analysed the ability of Cho-M3 cells to undergo apoptosis induced by the DNA damaging agent etoposide (Budd et al., 2004). In this scenario, the stimulation of M3 muscarinic receptor activated anti-apoptotic pathways through a mechanism that is independent of PLCβ activation and involves Bcl-2 upregulation. Also, the induction of apoptotic processes by DNA damage, oxidative stress and mitochondrial inhibition was reported to be reverted by muscarinic receptor activation in human neuroblastoma cells (De Sarno et al., 2003). However there is evidence that some of the anti-apoptotic pathways downstream the M3 receptor may be initiated independently of Gαq. An M3 receptor truncation mutant, d565-M3, that preserves the G protein activation capacity did not have this anti-apoptotic effect (Tobin & Budd, 2003). Thus, Gαq signalling downstream the M3 receptor in Cho cells would be responsible for cell growth arrest whereas apoptosis protection upon DNA damage might be mediated by β-arrestin-dependent pathways. In our hands, it was noteworthy that the combination of carbachol stimulation and Gαq overexpression lead to significant decreases in the cell index, which is indicative of cell death. This is consistent with the notion that overactivated Gαq promotes the transition

from survival/proliferative responses to apoptosis (Dhanasekaran et al., 1998). Indeed, muscarinic M3 activation with pilocarpine has been shown to promote apoptosis in human skin fibroblast cells, which correlated with JNK and caspase-3 activation (Reina et al., 2010). In our experimental setting, we observed that the slope of the cell index curve for Gαq cells is negative for approx. 48 hours following carbachol but it progressively increases afterwards. Thus, we suggest that, in a context of excessive signalling downstream the M3 muscarinic receptor, growth arrest responses might coexist with apoptosis events. Coincidently, it has been reported that M3 activation upon overexpression of Rac1 or a constitutively active Rac1 mutant results in apoptosis, whereas the expression of a dominant-negative form of Rac1 leads to a diminished cell growth inhibition (Shafer & Williams, 2004). Our results show that the ability to promote apoptosis differs between Gαq and Gαq-EEAA cells, suggesting a potential role for the Gαq/PKCζ complex in this process. In sum, the possibility that Gαq/PKCζ acts upstream Tiam1 in the activation of the Rac1/JNK pathways clearly deserves further investigation.

In order to confirm the involvement of the Gαq/PKCζ complex in apoptosis promotion, we studied cell death upon nutrient-starvation, an established model of apoptosis. Our results clearly show that Gαq overexpression promotes apoptosis entry with respect to control, whereas the Gαq-EEAA mutant fails to do so. We examined the different cell populations with a brightfield microscope after the slope in the growth curve of Gαq cells had turned negative. This showed cellular shrinkage and membrane blebbing in Gαq, but not in control or Gαq-EEAA cells, which reflected the boiling action of the membrane (zeiosis) characteristic of an apoptotic phenotype (Squier & Cohen, 2001). Indeed, in the absence of a clear molecular definition of apoptosis, this process can be defined by morphological markers such as those mentioned. The drastic change in the growth curve observed in all our experiments together with the homogeneous phenotypical changes visualised suggest that this is an event of programmed cell death (apoptosis) instead of accidental cell death (necrosis). This is also consistent with the reported role for Gαq in apoptosis (Adams et al., 2000). Additionally, preliminary results not shown in this thesis suggest that several molecular markers related to apoptosis are differentially regulated in Gαq and Gαq-EEAA populations. It seems that pro-survival protein Akt is dephosphorylated, and the proapoptotic protein Bad is upregulated, at an earlier stage in Gαq compared to Gαq-EEAA cells. Additionally, the performance of vital-dye exclusion assays and the determination of other biochemical markers would be necessary to further confirm the notion of apoptosis-related cell death in our experimental settings.

Evidences commented above suggest the possibility that the Gαq/PKCζ axis might activate the JNK cascade in the context of cell growth arrest and apoptosis (Fig. D5). In this line, several reports suggest that Gαq can promote apoptosis independently of its

classical effector PLCβ. Vascular smooth muscle cells (VSMC) that express activated Gαq undergo caspase activation and apoptosis. Although the precise apoptotic mechanisms are undefined, cell death is independent of PLCβ as inhibitors of IP_3- or PKC-dependent signalling did not block Gαq-induced apoptosis (Peavy et al., 2005). Since the EEAA mutation has been shown to partly affect PLCβ-mediated IP_3 generation, although it did not seem to affect ERK1/2 downstream signalling, we assessed whether PLCβ had a role in the apoptotic events observed in our system. We speculated that if diminished PLCβ signalling downstream the Gαq-EEAA mutant was responsible for its retarded apoptotic entry compared to Gαq-wt, a PLCβ inhibitor would balance the difference observed. However, apoptosis entry of the mutant Gαq population was still delayed with respect to Gαq-wild type cells. This strongly suggests that PLCβ is not involved in Gαq-mediated apoptosis responses in Cho cells. In addition to JNK, we should consider the possibility that the activation of ERK5 by the Gαq/PKCζ axis might contribute to apoptosis promotion. This might seem paradoxical in principle since ERK5 is a key pro-survival factor shown to inhibit apoptosis (Nithianandarajah-Jones et al., 2012), but it also has been found to promote apoptosis in certain contexts. Indeed, it was recently found that the ERK5 signalling axis positively regulates apoptosis of developing thymocytes and its activity correlates with the expression of Nur77, a family of orphan nuclear receptors (Sohn et al., 2008). The intrinsic function Nur77 has been characterized as cell type-dependent and agent context-dependent, which might explain why ERK5 plays a distinct role in thymocytes. Since ERK5 is to date the only pathway known to act downstream of the Gαq/PKCζ complex, a possible function in apoptosis deserves to be clarified.

Since we have described the occurrence of a Gαq/PKCζ signalling axis in the heart it is tempting to speculate that it might be important for apoptosis-related processes like heart failure. A role for PKCζ in apoptosis promotion in the heart has been previously proposed. Stimulation of the Gq-coupled thromboxane TxA2 receptor in isolated adult cardiac myocytes directly induces apoptosis which can be ascribed to PKCζ function (Shizukuda & Buttrick, 2002). In this process it seemed that PKCζ was required for the inhibition of the PI3K/Akt pathway downstream the TxA2 receptor. This is consistent with the reported negative role of Gαq on the Akt pathway (Ballou et al., 2000), which has also been shown to occur independently of PLCβ (Fan et al., 2003). Several reports suggest that Gαq-mediated apoptosis involves the inhibition of Akt signalling (Ben-Ami et al., 2011), and particularly in the heart this seems to involve the depletion of PIP_2 (Howes et al., 2003). Distinctive features in angiotensin II and Gαq overexpression-induced heart failure are the production of reactive oxygen species (ROS) and mitochondrial dysfunction. It is known that angiotensin stimulates NADPH oxidase (NOX), an enzyme responsible for ROS production in the cytoplasm (Mollnau, 2002). NOX-dependent ROS formation in turn stimulates mitochondrial ROS production, which leads to the dysfunction of mitochondria

(Zimmerman & Zucker, 2009). The first step for the activation of NOX is the translocation of the p47phox subunit to the plasma membrane which is followed by subsequent activation steps, including an interaction between p40phox and p67phox subunits through their PB1 domains (Wilson et al., 2003). Angiotensin II was reported to induce p47phox translocation in the heart, which was correlated with cardiac hypertrophy (Bendall et al., 2002). Notably, PKCζ has been previously shown to promote activatory phosphorylation and to colocalise with the p47phox subunit at the membrane (Frey, 2002). Thus, PKCζ might be the link between Gαq-mediated signalling and mitochondrial dysfunction in the heart leading to apoptosis, through the promotion of NOX activation. In summary, we propose two different potential mechanisms for the induction of apoptosis downstream the Gαq/PKCζ complex in the heart: Akt pathway downregulation and NOX pathway activation (Fig. D5).

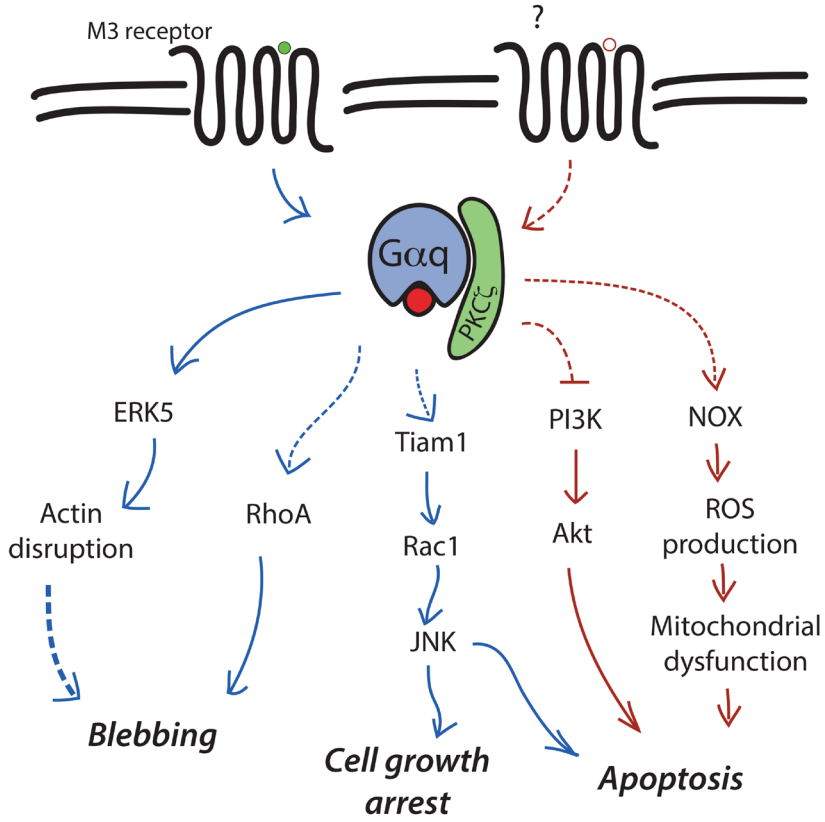

Figure D5. Possible pathways downstream the Gαq/PKCζ complex in blebbing, cell growth arrest and apoptosis. Graphical representation of potential downstream targets for the Gαq/PKCζ complex in muscarinic M3 receptor-induced blebbing and growth arrest/apoptosis (blue lines) and in Gαq-dependent activation of apoptosis (red lines). Punctuated lines are hypothetical links whereas solid lines are reported connections.

To summarise, we have described the involvement of the Gαq/PKCζ complex in muscarinic M3 receptor-induced blebbing and cell growth arrest and in nutrient-deprivation apoptosis. Further work is required for the integration of these preliminary evidences into a comprehensible biochemical model that explains the novel functions of PKCζ as a cellular effector of Gαq.

VI. CONCLUSIONS

1. The Gαq/PKCζ/ERK5 pathway is fully functional in cardiac myocytes and fibroblasts and in murine hearts *in vivo*.

2. ERK5 activation by Gq-GPCRs is Gαq-biased.

3. The Gαq/PKCζ complex can be detected in living cells and is highly specific.

4. PKCζ interacts with Gαq through its PB1 type II domain. Lysine 19 is a key aminoacid for this interaction. In turn, Gαq interacts with PKCζ through a pseudo-PB1 type I domain located within the effector-binding region. Glutamic acids at positions 234 and 245 are key determinants of the interaction.

5. The formation of an efficient Gαq/PKCζ complex is essential for ERK5 activation by Gq-coupled GPCRs.

6. GRK2 negatively regulates Gαq association to PKCζ and ERK5 activation by Gq-coupled GPCRs.

7. PKCζ transiently dimerises in response to Gq-GPCR stimulation and acts as a scaffold between Gαq and ERK5 upon activation of the G protein.

8. The Gαq/PKCζ complex is involved in membrane blebbing and cell growth arrest induced by muscarinic M3 receptor activation, and in the promotion of apoptosis.

VII. ABSTRACT

The cell is a dynamic entity with highly intertwined biochemical networks responding to internal and external perturbations in an orchestrated manner. The elements in those networks are usually proteins that build complex circuits through protein-protein interactions. G proteins are essential cellular components for the signalling events following G protein-coupled receptor (GPCR) activation. This function is achieved through specific and evolutionary conserved interactions between activated G proteins and a number of cellular effectors. We have recently reported that ERK5 activation by Gq-coupled GPCR requires participation of a novel Gαq effector, PKCζ. Here we present a biochemical and cellular characterization of the Gαq/PKCζ signalling axis. We demonstrate that the Gαq/PKCζ cascade is responsible for ERK5 activation in response to Angiotensin II in the heart. Also, it was show that this pathway displays Gαq-biased properties which has potentially interesting therapeutic implications. Next, we performed a cellular and biochemical characterization of the Gαq/PKCζ complex. We determined the occurrence and the specificity of the Gαq/PKCζ complex in living cells. Also, we identified lysine 19, located in the PB1 domain of PKCζ, and two glutamic acids at positions 234 and 245, located in a novel region of Gαq termed pseudo-PB1 domain, to be essential for the formation of the Gαq/PKCζ complex. Introduction of the double mutation (E234/E245-AA) in Gαq completely abrogated ERK5 activation, demonstrating requirement of an efficient Gαq/PKCζ complex for the activation of the pathway. Additionally, we found that GRK2, but not RGS2/4, prevents the association of PKCζ to Gαq due to sequestering of the G protein. This effect, together with receptor desensitisation, impaires the downstream activation of ERK5 by Gq-coupled GPCR. Next, we demostrated that PKCζ is not phosphorylated in response to Gq-coupled GPCR stimulation, but instead we provide the first evidence for a transient dimerisation of PKCζ. This protein was found to physically link activated Gαq and ERK5. Finally, we studied the role of the Gαq/PKCζ complex in Gαq-dependent cellular effects. The formation this protein complex was shown to be required for muscarinic M3 receptor-induced membrance blebbing and cell growth arrest. Additionally, the Gαq/PKCζ complex was found to be necessary for apoptosis promotion. Overall, this study provides important biochemical and cellular insight into Gq-coupled receptor signalling via the novel effector PKCζ.

IX. REFERENCES

Adams, J. W., Pagel, a. L., Means, C. K., Oksenberg, D., Armstrong, R. C., & Brown, J. H. 2000. Cardiomyocyte Apoptosis Induced by G q Signaling Is Mediated by Permeability Transition Pore Formation and Activation of the Mitochondrial Death Pathway. Circulation Research, 87(12): 1180–1187.

Adams, J. W., Sakata, Y., Davis, M. G., Sah, V. P., Wang, Y., Liggett, S. B., Chien, K. R., Brown, J. H., & Dorn, G. W. 1998. Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. Proceedings of the National Academy of Sciences of the United States of America, 95(17): 10140–5.

Ahn, S., Shenoy, S. K., Wei, H., & Lefkowitz, R. J. 2004. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. The Journal of biological chemistry, 279(34): 35518–25.

Balendran, A., Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., & Alessi, D. R. 2000. A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCζ) and PKC-related kinase 2 by PDK1. The Journal of biological chemistry, 275(27): 20806–13.

Ballou, L. M., Chattopadhyay, M., Li, Y., Scarlata, S., & Lin, R. Z. 2006. Galphaq binds to p110alpha/p85alpha phosphoinositide 3-kinase and displaces Ras. The Biochemical journal, 394(Pt 3): 557–62.

Ballou, L. M., Cross, M. E., Huang, S., McReynolds, E. M., Zhang, B. X., & Lin, R. Z. 2000. Differential regulation of the phosphatidylinositol 3-kinase/Akt and p70 S6 kinase pathways by the alpha(1A)-adrenergic receptor in rat-1 fibroblasts. The Journal of biological chemistry, 275(7): 4803–9.

Ballou, L. M., Lin, H.-Y., Fan, G., Jiang, Y.-P., & Lin, R. Z. 2003. Activated G alpha q inhibits p110 alpha phosphatidylinositol 3-kinase and Akt. The Journal of biological chemistry, 278(26): 23472–9.

Bansal, G., Druey, K. M., & Xie, Z. 2007. R4 RGS proteins: regulation of G-protein signaling and beyond. Pharmacology & therapeutics, 116(3): 473–95.

Barros, J. C., & Marshall, C. J. 2005. Activation of either ERK1/2 or ERK5 MAP kinase pathways can lead to disruption of the actin cytoskeleton. Journal of cell science, 118(Pt 8): 1663–71.

Ben-Ami, I., Yao, Z., Naor, Z., & Seger, R. 2011. Gαq protein-induced apoptosis is mediated by AKT kinase inhibition that leads to protein kinase C-induced c-Jun N-terminal kinase activation. The Journal of biological chemistry, 286(35): 31022–31.

Bence, K., Ma, W., Kozasa, T., & Huang, X. Y. 1997. Direct stimulation of Bruton's tyrosine kinase by G(q)-protein alpha-subunit. Nature, 389(6648): 296–9.

Bendall, J. K., Cave, A. C., Heymes, C., Gall, N., & Shah, A. M. 2002. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. Circulation, 105(3): 293–6.

Berra, E., Díaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sánchez, P., Sanz, L., & Moscat, J. 1995. Evidence for a role of MEK and MAPK during signal transduction by protein kinase C zeta. The EMBO journal, 14(24): 6157–63.

Berts, a, Zhong, H., & Minneman, K. P. 1999. No role for Ca++ or protein kinase C in alpha-1A adrenergic receptor activation of mitogen-activated protein kinase pathways in transfected PC12 cells. Molecular pharmacology, 55(2): 296–303.

Bhatnagar, A., Sheffler, D. J., Kroeze, W. K., Compton-Toth, B., & Roth, B. L. 2004. Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly modulates the signaling of selected Galphaq-coupled protein receptors. The Journal of biological chemistry, 279(33): 34614–23.

Booden, M., Siderovski, D., & JD, C. 2002. Leukemia-associated Rho guanine nucleotide exchange factor promotes Gq-coupled activation of RhoA. Molecular and cellular biology, 22(12): 4053–4061.

Borges, L., Bigarella, C. L., Baratti, M. O., Crosara-Alberto, D. P., Joazeiro, P. P., Franchini, K. G., Costa, F. F., & Saad, S. T. O. 2008. ARHGAP21 associates with FAK and PKCζ and is redistributed after cardiac pressure overload. Biochemical and biophysical research communications, 374(4): 641–6.

Bos, J. L., Rehmann, H., & Wittinghofer, A. 2007. GEFs and GAPs: critical elements in the control of small G proteins. Cell, 129(5): 865–77.

Bouzegrhane, F., & Thibault, G. 2002. Is angiotensin II a proliferative factor of cardiac fibroblasts? Cardiovascular research, 53(2): 304–12.

Bowman, J. C., Steinberg, S. F., Jiang, T., Geenen, D. L., Fishman, G. I., & Buttrick, P. M. 1997. Expression of protein kinase C beta in the heart causes hypertrophy in adult mice and sudden death in neonates. The Journal of clinical investigation, 100(9): 2189–95.

Braun, M., & Mochly-Rosen, D. 2003. Opposing effects of δ- and ζ-protein kinase C isozymes on cardiac fibroblast proliferation: use of isozyme-selective inhibitors. Journal of Molecular and Cellular Cardiology, 35(8): 895–903.

Braz, J. C., Gregory, K., Pathak, A., Zhao, W., Sahin, B., Klevitsky, R., Kimball, T. F., Lorenz, J. N., Nairn, A. C., Liggett, S. B., Bodi, I., Wang, S., Schwartz, A., Lakatta, E. G., DePaoli-Roach, A. A., Robbins, J., Hewett, T. E., Bibb, J. A., Westfall, M. V, Kranias, E. G., et al. 2004. PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nature medicine, 10(3): 248–54.

Buchsbaum, R. J., Connolly, B. A., & Feig, L. A. 2002. Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Molecular and cellular biology, 22(12): 4073–85.

Budd, D. C., Rae, a, & Tobin, a B. 1999. Activation of the mitogen-activated protein kinase pathway by a Gαq/11-coupled muscarinic receptor is independent of receptor internalization. The Journal of biological chemistry, 274(18): 12355–60.

Budd, D. C., Spragg, E. J., Ridd, K., & Tobin, A. B. 2004. Signalling of the M3-muscarinic receptor to the anti-apoptotic pathway. The Biochemical journal, 381(Pt 1): 43–9.

Burdon, D., Patel, R., Challiss, R., & Blank, J. 2002. Growth inhibition by the muscarinic M (3) acetylcholine receptor: evidence for p21 (Cip1/Waf1) involvement in G (1) arrest. Biochemical Journal, 559: 549–559.

Carman, C. V, Lisanti, M. P., & Benovic, J. L. 1999a. Regulation of G protein-coupled receptor kinases by caveolin. The Journal of biological chemistry, 274(13): 8858–64.

Carman, C. V, Parent, J. L., Day, P. W., Pronin, a N., Sternweis, P. M., Wedegaertner, P. B., Gilman, a G., Benovic, J. L., & Kozasa, T. 1999b. Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. The Journal of biological chemistry, 274(48): 34483–92.

Cartier, A., Parent, A., Labrecque, P., Laroche, G., & Parent, J.-L. 2011. WDR36 acts as a scaffold protein tethering a G-protein-coupled receptor, G{alpha}q and phospholipase C{beta} in a signalling complex. Journal of cell science, 124(Pt 19): 3292–3304.

Caulfield, M. P. 1993. Muscarinic receptors--characterization, coupling and function. Pharmacology & therapeutics, 58(3): 319–79.

Changeux, J. P., Kasai, M., & Lee, C. Y. 1970. Use of a snake venom toxin to characterize the cholinergic receptor protein. Proceedings of the National Academy of Sciences of the United States of America, 67(3): 1241–7.

Charras, G. T. 2008. A short history of blebbing. Journal of microscopy, 231(3): 466–78.

Chernyavsky, A. I., Arredondo, J., Wess, J., Karlsson, E., & Grando, S. A. 2004. Novel signaling pathways mediating reciprocal control of keratinocyte migration and wound epithelialization through M3 and M4 muscarinic receptors. The Journal of cell biology, 166(2): 261–72.

Chikumi, H., Vázquez-Prado, J., Servitja, J.-M., Miyazaki, H., & Gutkind, J. S. 2002. Potent activation of RhoA by Galpha q and Gq-coupled receptors. The Journal of biological chemistry, 277(30): 27130–4.

Churchill, E., Budas, G., Vallentin, A., Koyanagi, T., & Mochly-Rosen, D. 2008. PKC isozymes in chronic cardiac disease: possible therapeutic targets? Annual review of pharmacology and toxicology, 48: 569–99.

Ciruela, F., & McIlhinney, R. A. 2001. Metabotropic glutamate receptor type 1alpha and tubulin assemble into dynamic interacting complexes. Journal of neurochemistry, 76(3): 750–7.

Coghlan, M. P., Chou, M. M., & Carpenter, C. L. 2000. Atypical protein kinases Clambda and -zeta associate with the GTP-binding protein Cdc42 and mediate stress fiber loss. Molecular and cellular biology, 20(8): 2880–9.

Coso, O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J., & Gutkind, J. S. 1995. Transforming G protein-coupled receptors potently activate JNK (SAPK). Evidence for a divergence from the tyrosine kinase signaling pathway. The Journal of biological chemistry, 270(10): 5620–4.

Cude, K., Wang, Y., Choi, H.-J., Hsuan, S.-L., Zhang, H., Wang, C.-Y., & Xia, Z. 2007. Regulation of the G2-M cell cycle progression by the ERK5-NFkappaB signaling pathway. The Journal of cell biology, 177(2): 253–64.

Dai, D.-F., Johnson, S. C., Villarin, J. J., Chin, M. T., Nieves-Cintrón, M., Chen, T., Marcinek, D. J., Dorn, G. W., Kang, Y. J., Prolla, T. a, Santana, L. F., & Rabinovitch, P. S. 2011. Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphaq overexpression-induced heart failure. Circulation research, 108(7): 837–46.

Dave, R. H., Saengsawang, W., Yu, J.-Z., Donati, R., & Rasenick, M. M. 2009. Heterotrimeric G-proteins interact directly with cytoskeletal components to modify microtubule-dependent cellular processes. Neuro-Signals, 17(1): 100–8.

Day, P. W., Carman, C. V, Sterne-Marr, R., Benovic, J. L., & Wedegaertner, P. B. 2003. Differential interaction of GRK2 with members of the G alpha q family. Biochemistry, 42(30): 9176–84.

De Lean, A., Stadel, J. M., & Lefkowitz, R. J. 1980. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. The Journal of biological chemistry, 255(15): 7108–17.

De Sarno, P., Shestopal, S. a, King, T. D., Zmijewska, A., Song, L., & Jope, R. S. 2003. Muscarinic receptor activation protects cells from apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition. The Journal of biological chemistry, 278(13): 11086–93.

Deacon, K., & Blank, J. L. 1999. MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. The Journal of biological chemistry, 274(23): 16604–10.

Decock, J. B., Gillespie-Brown, J., Parker, P. J., Sugden, P. H., & Fuller, S. J. 1994. Classical, novel and atypical isoforms of PKC stimulate ANF- and TRE/AP-1-regulated-promoter activity in ventricular cardiomyocytes. FEBS letters, 356(2-3): 275–8.

DeWire, S. M., Ahn, S., Lefkowitz, R. J., & Shenoy, S. K. 2007. β-Arrestins and Cell Signaling. Annual Review of Physiology, 69(1): 483–510.

Dhanasekaran, N., Tsim, S. T., Dermott, J. M., & Onesime, D. 1998. Regulation of cell proliferation by G proteins. Oncogene, 17(11 Reviews): 1383–94.

Diaz-Meco, M. T., & Moscat, J. 2001. MEK5, a new target of the atypical protein kinase C isoforms in mitogenic signaling. Molecular and Cellular Biology, 21(4): 1218.

Diaz-Meco, M. T., & Moscat, J. 2012. The atypical PKCs in inflammation: NF-κB and beyond. Immunological reviews, 246(1): 154–67.

Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., & Schlessinger, J. 1996. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature, 383(6600): 547–50.

Disatnik, M. H., Buraggi, G., & Mochly-Rosen, D. 1994. Localization of protein kinase C isozymes in cardiac myocytes. Experimental cell research, 210(2): 287–97.

Dorn, G., & Force, T. 2005. Protein kinase cascades in the regulation of cardiac hypertrophy. Journal of Clinical Investigation, 115(3): 537–537.

Drin, G., & Scarlata, S. 2007. Stimulation of phospholipase Cβ by membrane interactions, interdomain movement, and G protein binding — How many ways can you activate an enzyme? Cellular Signalling, 19(7): 1383–1392.

Dwivedi, P. P., Hii, C. S. T., Ferrante, A., Tan, J., Der, C. J., Omdahl, J. L., Morris, H. A., & May, B. K. 2002. Role of MAP kinases in the 1,25-dihydroxyvitamin D3-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. The Journal of biological chemistry, 277(33): 29643–53.

Díaz-Meco, M. T., Municio, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L., & Moscat, J. 1996. The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. Cell, 86(5): 777–86.

Echeverria, V., Hinrichs, M. V., Torrejon, M., Ropero, S., Martinez, J., Toro, M. J., & Olate, J. 2000. Mutagenesis in the switch IV of the helical domain of the human Gs alpha reduces its GDP/GTP exchange rate. Journal of Cellular Biochemistry, 76(3): 368–375.

Esparís-Ogando, A., Díaz-Rodríguez, E., Montero, J. C., Yuste, L., Crespo, P., & Pandiella, A. 2002. Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells overexpressing ErbB2. Molecular and cellular biology, 22(1): 270–85.

Etienne-Manneville, S., & Hall, A. 2002. Rho GTPases in cell biology. Nature, 420(6916): 629–35.

Fackler, O. T., & Grosse, R. 2008. Cell motility through plasma membrane blebbing. The Journal of cell biology, 181(6): 879–84.

Fan, G., Ballou, L. M., & Lin, R. Z. 2003. Phospholipase C-independent activation of glycogen synthase kinase-3beta and C-terminal Src kinase by Galphaq. The Journal of biological chemistry, 278(52): 52432–6.

Fan, G., Jiang, Y.-P., Lu, Z., Martin, D. W., Kelly, D. J., Zuckerman, J. M., Ballou, L. M., Cohen, I. S., & Lin, R. Z. 2005. A transgenic mouse model of heart failure using inducible Galpha q. The Journal of biological chemistry, 280(48): 40337–46.

Faure, M., Voyno-Yasenetskaya, T. A., & Bourne, H. R. 1994. cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. The Journal of biological chemistry, 269(11): 7851–4.

Ferguson, S. S. G. 2007. Phosphorylation-independent attenuation of GPCR signalling. Trends in Pharmacological Sciences, 28(4): 173–179.

Fielitz, J., Kim, M.-S., Shelton, J. M., Qi, X., Hill, J. A., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. 2008. Requirement of protein kinase D1 for pathological cardiac remodeling. Proceedings of the National Academy of Sciences of the United States of America, 105(8): 3059–63.

Filtz, T. M., Grubb, D. R., McLeod-Dryden, T. J., Luo, J., & Woodcock, E. a. 2009. Gαq-initiated cardiomyocyte hypertrophy is mediated by phospholipase Cbeta1b. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 23(10): 3564–70.

Frey, N., & Olson, E. N. 2003. Cardiac hypertrophy: the good, the bad, and the ugly. Annual review of physiology, 65: 45–79.

Frey, R. S. 2002. PKCζ Regulates TNF-αlpha-Induced Activation of NADPH Oxidase in Endothelial Cells. Circulation Research, 90(9): 1012–1019.

Fukuhara, S., Marinissen, M. J., Chiariello, M., & Gutkind, J. S. 2000. Signaling from G protein-coupled receptors to ERK5/Big MAPK 1 involves Galpha q and Galpha 12/13 families of heterotrimeric G proteins. Evidence for the existence of a novel Ras AND Rho-independent pathway. The Journal of biological chemistry, 275(28): 21730–6.

Furchgott, R. F. 1964. Receptor Mechanisms. Annual Review of Pharmacology, 4(1): 21–50.

Garcia-Guerra, L., Nieto-Vazquez, I., Vila-Bedmar, R., Jurado-Pueyo, M., Zalba, G., Díez, J., Murga, C., Fernández-Veledo, S., Mayor, F., & Lorenzo, M. 2010. G protein-coupled receptor kinase 2 plays a relevant role in insulin resistance and obesity. Diabetes, 59(10): 2407–17.

García-Hoz, C., Sánchez-Fernández, G., Díaz-Meco, M. T., Moscat, J., Mayor, F., & Ribas, C. 2010. G alpha(q) acts as an adaptor protein in protein kinase C zeta (PKCζ)-mediated ERK5 activation by G protein-coupled receptors (GPCR). The Journal of biological chemistry, 285(18): 13480–9.

García-Hoz, C., Sánchez-Fernández, G., García-Escudero, R., Fernández-Velasco, M., Palacios-García, J., Ruiz-Meana, M., Díaz-Meco, M. T., Leitges, M., Moscat, J., García-Dorado, D., Boscá, L., Mayor, F., & Ribas, C. 2012. Protein Kinase C (PKC)ζ-mediated Gαq Stimulation of ERK5 Protein Pathway in Cardiomyocytes and Cardiac Fibroblasts. The Journal of biological chemistry, 287(10): 7792–802.

Geetha, T., & Wooten, M. W. 2003. Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. The Journal of biological chemistry, 278(7): 4730–9.

Giannessi, D., Del Ry, S., & Vitale, R. L. 2001. The role of endothelins and their receptors in heart failure. Pharmacological research : the official journal of the Italian Pharmacological Society, 43(2): 111–26.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. Annual review of biochemistry, 56: 615–49.

Godin, C., & Ferguson, S. 2010. The angiotensin II type 1 receptor induces membrane blebbing by coupling to Rho A, Rho kinase, and myosin light chain kinase. Molecular Pharmacology, 77(6): 903–911.

Goldberg, M., & Steinberg, S. F. 1996. Tissue-specific developmental regulation of protein kinase C isoforms. Biochemical pharmacology, 51(8): 1089–93.

Goldsmith, Z. G., & Dhanasekaran, D. N. 2007. G protein regulation of MAPK networks. Oncogene, 26(22): 3122–42.

Golebiewska, U., & Scarlata, S. 2008. G{alpha}q Binds Two Effectors Separately in Cells: Evidence for Predetermined Signaling Pathways. Biophys. J., 95(5): 2575–2582.

Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V, Penn, R. B., Gagnon, A. W., Keen, J. H., & Benovic, J. L. 1996. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature, 383(6599): 447–50.

Graybill, C., Wee, B., Atwood, S. X., & Prehoda, K. E. 2012. Partitioning-defective protein 6 (Par-6) activates atypical protein kinase C (aPKC) by pseudosubstrate displacement. The Journal of biological chemistry, 287(25): 21003–11.

Gudermann, T., Schöneberg, T., & Schultz, G. 1997. Functional and structural complexity of signal transduction via G-protein-coupled receptors. Annual review of neuroscience, 20: 399–427.

Gutkind, J. S., Novotny, E. a, Brann, M. R., & Robbins, K. C. 1991. Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. Proceedings of the National Academy of Sciences of the United States of America, 88(11): 4703–7.

Hahn, H. S., Marreez, Y., Odley, A., Sterbling, A., Yussman, M. G., Hilty, K. C., Bodi, I., Liggett, S. B., Schwartz, A., & Dorn, G. W. 2003. Protein kinase Calpha negatively regulates systolic and diastolic function in pathological hypertrophy. Circulation research, 93(11): 1111–9.

Hajduch, E., Turban, S., Le Liepvre, X., Le Lay, S., Lipina, C., Dimopoulos, N., Dugail, I., & Hundal, H. S. 2008. Targeting of PKCζ and PKB to caveolin-enriched microdomains represents a crucial step underpinning the disruption in PKB-directed signalling by ceramide. The Biochemical journal, 410(2): 369–79.

Harden, T. K., Waldo, G. L., Hicks, S. N., & Sondek, J. 2011. Mechanism of activation and inactivation of Gαq/phospholipase C-β signaling nodes. Chemical reviews, 111(10): 6120–9.

Harris, I. S., Treskov, I., Rowley, M. W., Heximer, S., Kaltenbronn, K., Finck, B. N., Gross, R. W., Kelly, D. P., Blumer, K. J., & Muslin, A. J. 2004. G-protein signaling participates in the development of diabetic cardiomyopathy. Diabetes, 53(12): 3082–90.

Hayashi, M. 2004. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. Journal of Clinical Investigation, 113(8):1138-48.

Hayashi, M., Kim, S.-W., Imanaka-Yoshida, K., Yoshida, T., Abel, E. D., Eliceiri, B., Yang, Y., Ulevitch, R. J., & Lee, J.-D. 2004. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. The Journal of clinical investigation, 113(8): 1138–48.

Hayashi, M., & Lee, J.-D. 2004. Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. Journal of molecular medicine (Berlin, Germany), 82(12): 800–8.

Heasley, L., & Storey, B. 1996. GTPase-deficient G alpha 16 and G alpha q induce PC12 cell differentiation and persistent activation of cJun NH2-terminal kinases. Molecular and cellular biology, 16(2): 648.

Hein, L., Stevens, M. E., Barsh, G. S., Pratt, R. E., Kobilka, B. K., & Dzau, V. J. 1997. Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. Proceedings of the National Academy of Sciences of the United States of America, 94(12): 6391–6.

Hepler, J. R., & Gilman, A. G. 1992. G proteins. Trends in biochemical sciences, 17(10): 383–7.

Hewavitharana, T., & Wedegaertner, P. B. 2011. Non-canonical signaling and localizations of heterotrimeric G proteins. Cellular signalling, 24(1): 25–34.

Hirano, Y., Yoshinaga, S., Ogura, K., Yokochi, M., Noda, Y., Sumimoto, H., & Inagaki, F. 2004. Solution structure of atypical protein kinase C PB1 domain and its mode of interaction with ZIP/p62 and MEK5. The Journal of biological chemistry, 279(30): 31883–90.

Hirano, Y., Yoshinaga, S., Takeya, R., Suzuki, N. N., Horiuchi, M., Kohjima, M., Sumimoto, H., & Inagaki, F. 2005. Structure of a cell polarity regulator, a complex between atypical PKC and Par6 PB1 domains. The Journal of biological chemistry, 280(10): 9653–61.

Hislop, J. N., & Von Zastrow, M. 2010. Role of Ubiquitination in Endocytic Trafficking of G-Protein-Coupled Receptors. Traffic (Copenhagen, Denmark), 12(2): 137–48.

Howes, A. L., Arthur, J. F., Zhang, T., Miyamoto, S., Adams, J. W., Dorn, G. W., Woodcock, E. a, & Brown, J. H. 2003. Akt-mediated cardiomyocyte survival pathways are compromised by G alpha q-induced phosphoinositide 4,5-bisphosphate depletion. The Journal of biological chemistry, 278(41): 40343–51.

Hoyer, D. P., Korkmaz, Y., Grönke, S., Addicks, K., Wettschureck, N., Offermanns, S., & Reuter, H. 2010. Differential expression of protein kinase C isoforms in coronary arteries of diabetic mice lacking the G-protein Gα11. Cardiovascular diabetology, 9(1): 93.

Hubbard, K. B., & Hepler, J. R. 2006. Cell signalling diversity of the Gαqalpha family of heterotrimeric G proteins. Cellular signalling, 18(2): 135–50.

Hunter, J. J., & Chien, K. R. 1999. Signaling pathways for cardiac hypertrophy and failure. The New England journal of medicine, 341(17): 1276–83.

Hunyady, L., & Catt, K. J. 2006. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. Molecular endocrinology (Baltimore, Md.), 20(5): 953–70.

Ibarrondo, J., Joubert, D., Dufour, M. N., Cohen-Solal, A., Homburger, V., Jard, S., & Guillon, G. 1995. Close association of the alpha subunits of Gαq and Gα11 G proteins with actin filaments in WRK1 cells: relation to G protein-mediated phospholipase C activation. Proceedings of the National Academy of Sciences of the United States of America, 92(18): 8413–17.

Ieda, M., Tsuchihashi, T., Ivey, K. N., Ross, R. S., Hong, T.-T., Shaw, R. M., & Srivastava, D. 2009. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. Developmental cell, 16(2): 233–44.

Ikeda, Y., Aihara, K.-I., Sato, T., Akaike, M., Yoshizumi, M., Suzaki, Y., Izawa, Y., Fujimura, M., Hashizume, S., Kato, M., Yagi, S., Tamaki, T., Kawano, H., Matsumoto, T., Azuma, H., Kato, S., & Matsumoto, T. 2005. Androgen receptor gene knockout male mice exhibit impaired cardiac growth and exacerbation of angiotensin II-induced cardiac fibrosis. The Journal of biological chemistry, 280(33): 29661–6.

Imamura, T., & Vollenweider, P. 1999. G alpha-q/11 protein plays a key role in insulin-induced glucose transport in 3T3-L1 adipocytes. Molecular and cellular biology, 19(10): 6765.

Ito, T., Matsui, Y., Ago, T., Ota, K., & Sumimoto, H. 2001. Novel modular domain PB1 recognizes PC motif to mediate functional protein-protein interactions. The EMBO journal, 20(15): 3938–46.

Jeong, D., Cha, H., Kim, E., Kang, M., Yang, D. K., Kim, J. M., Yoon, P. O., Oh, J. G., Bernecker, O. Y., Sakata, S., Le, T. T., Cui, L., Lee, Y.-H., Kim, D. H., Woo, S.-H., Liao, R., Hajjar, R. J., & Park, W. J. 2006. PICOT inhibits cardiac hypertrophy and enhances ventricular function and cardiomyocyte contractility. Circulation research, 99(3): 307–14.

Johnson, G. J., Leis, L. A., & Dunlop, P. C. 1996. Specificity of G alpha q and G alpha 11 gene expression in platelets and erythrocytes. Expressions of cellular differentiation and species differences. The Biochemical journal, 318 (Pt 3: 1023–31.

Kacimi, R., & Gerdes, A. M. 2003. Alterations in G protein and MAP kinase signaling pathways during cardiac remodeling in hypertension and heart failure. Hypertension, 41(4): 968–77.

Kakkar, R., & Lee, R. T. 2010. Intramyocardial fibroblast myocyte communication. Circulation research, 106(1): 47–57.

Kan, Z., Jaiswal, B. S., Stinson, J., Janakiraman, V., Bhatt, D., Stern, H. M., Yue, P., Haverty, P. M., Bourgon, R., Zheng, J., Moorhead, M., Chaudhuri, S., Tomsho, L. P., Peters, B. a, Pujara, K., Cordes, S., Davis, D. P., Carlton, V. E. H., Yuan, W., Li, L., et al. 2010. Diverse somatic mutation patterns and pathway alterations in human cancers. Nature, 466(7308): 869–73.

Kasler, H. G., Victoria, J., Duramad, O., & Winoto, A. 2000. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Molecular and cellular biology, 20(22): 8382–9.

Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J., & Lee, J. D. 1998. Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature, 395(6703): 713–6.

Ke, N., Wang, X., Xu, X., & Abassi, Y. A. 2011. The xCELLigence System for Real-Time and Label-Free Monitoring of Cell Viability. (M. J. Stoddart, Ed.)Methods in Molecular Biology, 740(Mammalian Cell Viability: Methods and Protocols,): 33–43.

Kehat, I., Davis, J., Tiburcy, M., Accornero, F., Saba-El-Leil, M. K., Maillet, M., York, A. J., Lorenz, J. N., Zimmermann, W. H., Meloche, S., & Molkentin, J. D. 2011. Extracellular signal-regulated kinases 1 and 2 regulate the balance between eccentric and concentric cardiac growth. Circulation research, 108(2): 176–83.

Kehat, I., & Molkentin, J. D. 2010. Molecular pathways underlying cardiac remodeling during pathophysiological stimulation. Circulation, 122(25): 2727–35.

Keys, J. R., Greene, E. A., Koch, W. J., & Eckhart, A. D. 2002. Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. Hypertension, 40(5): 660–6.

Kim, G.-Y., Nigro, P., Fujiwara, K., Abe, J.-I., & Berk, B. C. 2012. p62 Binding to Protein Kinase C ζ Regulates Tumor Necrosis Factor α-Induced Apoptotic Pathway in Endothelial Cells. Arteriosclerosis, thrombosis, and vascular biology, 32(12): 2974–2980.

Kimura, T. E., Jin, J., Zi, M., Prehar, S., Liu, W., Oceandy, D., Abe, J., Neyses, L., Weston, A. H., Cartwright, E. J., & Wang, X. 2010. Targeted deletion of the extracellular signal-regulated protein kinase 5 attenuates hypertrophic response and promotes pressure overload-induced apoptosis in the heart. Circulation research, 106(5): 961–70.

Klein, S., Reuveni, H., & Levitzki, A. 2000. Signal transduction by a nondissociable heterotrimeric yeast G protein. Proceedings of the National Academy of Sciences, 97(7): 3219–3223.

Kleppisch, T., Voigt, V., Allmann, R., & Offermanns, S. 2001. G(alpha)q-deficient mice lack metabotropic glutamate receptor-dependent long-term depression but show normal long-term potentiation in the hippocampal CA1 region. The Journal of neuroscience : the official journal of the Society for Neuroscience, 21(14): 4943–8.

Kobilka, B. K., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Dixon, R. A., Keller, P., Caron, M. G., & Lefkowitz, R. J. 1987. Delineation of the intronless nature of the genes for the human and hamster beta 2-adrenergic receptor and their putative promoter regions. The Journal of biological chemistry, 262(15): 7321–7.

Koch, W. J., Lefkowitz, R. J., Milano, C. A., Akhter, S. A., & Rockman, H. A. 1998. Myocardial overexpression of adrenergic receptors and receptor kinases. Advances in pharmacology (San Diego, Calif.), 42: 502–6.

Kohout, T. A., Nicholas, S. L., Perry, S. J., Reinhart, G., Junger, S., & Struthers, R. S. 2004. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. The Journal of biological chemistry, 279(22): 23214–22.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D., & Rapp, U. R. 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature, 364(6434): 249–52.

Kong, K. C., Butcher, A. J., McWilliams, P., Jones, D., Wess, J., Hamdan, F. F., Werry, T., Rosethorne, E. M., Charlton, S. J., Munson, S. E., Cragg, H. a, Smart, A. D., & Tobin, A. B. 2010a. M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1. Proceedings of the National Academy of Sciences of the United States of America, 107(49): 21181–6.

Kong, K. C., Butcher, A. J., McWilliams, P., Jones, D., Wess, J., Hamdan, F. F., Werry, T., Rosethorne, E. M., Charlton, S. J., Munson, S. E., Cragg, H. a, Smart, A. D., & Tobin, A. B. 2010b. M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1. Proceedings of the National Academy of Sciences of the United States of America, 107(49): 21181–6.

Langley, J. N. 1901. Observations on the physiological action of extracts of the suprarenal bodies. The Journal of physiology, 27(3): 237–56.

Lappano, R., & Maggiolini, M. 2011. G protein-coupled receptors: novel targets for drug discovery in cancer. Nature Reviews Drug Discovery, 10(1): 47–60.

Lefkowitz, R. J. 2004. Historical review: a brief history and personal retrospective of seven-transmembrane receptors. Trends in pharmacological sciences, 25(8): 413–22.

Leitges, M., Sanz, L., Martin, P., Duran, a, Braun, U., García, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., & Moscat, J. 2001. Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. Molecular cell, 8(4): 771–80.

Liggett, S. 2006. Cardiac 7-transmembrane-spanning domain receptor portfolios: diversify, diversify, diversify. Journal of Clinical Investigation, 116(4): 1–3.

Limame, R., Wouters, A., Pauwels, B., Fransen, E., Peeters, M., Lardon, F., De Wever, O., & Pauwels, P. 2012. Comparative analysis of dynamic cell viability, migration and invasion assessments by novel real-time technology and classic endpoint assays. PloS one, 7(10): e46536.

Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, a G., & Mumby, S. M. 1993. Lipid modifications of G proteins: alpha subunits are palmitoylated. Proceedings of the National Academy of Sciences of the United States of America, 90(8): 3675–9.

Lindvall, J. M., Blomberg, K. E. M., Väliaho, J., Vargas, L., Heinonen, J. E., Berglöf, A., Mohamed, A. J., Nore, B. F., Vihinen, M., & Smith, C. I. E. 2005. Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. Immunological reviews, 203: 200–15.

Linseman, D. a, Hofmann, F., & Fisher, S. K. 2000. A role for the small molecular weight GTPases, Rho and Cdc42, in muscarinic receptor signaling to focal adhesion kinase. Journal of neurochemistry, 74(5): 2010–20.

Linseman, D. A., Benjamin, C. W., & Jones, D. A. 1995. Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells. The Journal of biological chemistry, 270(21): 12563–8.

Little, K. D., Hemler, M. E., & Stipp, C. S. 2004. Dynamic Regulation of a GPCR-Tetraspanin-G Protein Complex on Intact Cells : Central Role of CD81 in Facilitating GPR56-G alphaq / 11 Association, 15(May): 2375–2387.

Liu, L., Zhao, H., & Zuo, J. 2006. Protein kinase Czeta mediates insulin-induced glucose transport through actin remodeling in L6 muscle cells. Molecular biology of the cell, 17(May): 2322–2330.

Liu, S., Carrillo, J. J., Pediani, J. D., & Milligan, G. 2002. Effective information transfer from the alpha 1b-adrenoceptor to Galpha 11 requires both beta/gamma interactions and an aromatic group four amino acids from the C terminus of the G protein. The Journal of biological chemistry, 277(28): 25707–14.

Liu, X.-J., Yang, C., Gupta, N., Zuo, J., Chang, Y.-S., & Fang, F.-D. 2007. Protein kinase C-zeta regulation of GLUT4 translocation through actin remodeling in CHO cells. Journal of molecular medicine (Berlin, Germany), 85(8): 851–61.

Liu, Y. H., Yang, X. P., Sharov, V. G., Nass, O., Sabbah, H. N., Peterson, E., & Carretero, O. A. 1997. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. The Journal of clinical investigation, 99(8): 1926–35.

Lohse, M. J. 2010. Dimerization in GPCR mobility and signaling. Current opinion in pharmacology, 10(1): 53–8.

Lorenz, K., Schmitt, J. P., Schmitteckert, E. M., & Lohse, M. J. 2009. A new type of ERK1/2 autophosphorylation causes cardiac hypertrophy. Nature medicine, 15(1): 75–83.

Lutz, S., Freichel-Blomquist, A., Yang, Y., Rümenapp, U., Jakobs, K. H., Schmidt, M., & Wieland, T. 2005. The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gαq/11-coupled receptor signaling and RhoA. The Journal of biological chemistry, 280(12): 11134–9.

Lutz, S., Shankaranarayanan, A., Coco, C., Ridilla, M., Nance, M. R., Vettel, C., Baltus, D., Evelyn, C. R., Neubig, R. R., Wieland, T., & Tesmer, J. J. G. 2007. Structure of Galphaq-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science (New York, N.Y.), 318(5858): 1923–7.

Lyon, A. M., Tesmer, V. M., Dhamsania, V. D., Thal, D. M., Gutierrez, J., Chowdhury, S., Suddala, K. C., Northup, J. K., & Tesmer, J. J. G. 2011. An autoinhibitory helix in the C-terminal region of phospholipase C-β mediates Gαq activation. Nature structural & molecular biology, 18(9): 999–1005.

Ma, Y. C., & Huang, X. Y. 1998. Identification of the binding site for Gαq on its effector Bruton ' s tyrosine kinase. Proceedings of the National Academy of Sciences, 95(21): 12197.

Macara, I. G. 2004. Par proteins: partners in polarization. Current biology : CB, 14(4): R160–2.

Mackay, H. J., & Twelves, C. J. 2007. Targeting the protein kinase C family: are we there yet? Nature reviews. Cancer, 7(7): 554–62.

Magalhaes, A. C., Dunn, H., & Ferguson, S. S. G. 2012. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. British journal of pharmacology, 165(6): 1717–36.

Malhotra, R., D'Souza, K. M., Staron, M. L., Birukov, K. G., Bodi, I., & Akhter, S. a. 2010. G alpha(q)-mediated activation of GRK2 by mechanical stretch in cardiac myocytes: the role of protein kinase C. The Journal of biological chemistry, 285(18): 13748–60.

Marianayagam, N. J., Sunde, M., & Matthews, J. M. 2004. The power of two: protein dimerization in biology. Trends in biochemical sciences, 29(11): 618–25.

Mariggiò, S., García-Hoz, C., Sarnago, S., De Blasi, A., Mayor, F., & Ribas, C. 2006. Tyrosine phosphorylation of G-protein-coupled-receptor kinase 2 (GRK2) by c-Src modulates its interaction with Galphaq. Cellular signalling, 18(11): 2004–12.

Marinissen, M. J., Chiariello, M., Pallante, M., & Gutkind, J. S. 1999. A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. Molecular and cellular biology, 19(6): 4289–301.

Markou, T., Cieslak, D., Gaitanaki, C., & Lazou, A. 2009. Differential roles of MAPKs and MSK1 signalling pathways in the regulation of c-Jun during phenylephrine-induced cardiac myocyte hypertrophy. Molecular and cellular biochemistry, 322(1-2): 103–12.

Marty, C., Kozasa, T., Quinn, M. T., & Ye, R. D. 2006. Activation state-dependent interaction between Galphai and p67phox. Molecular and cellular biology, 26(13): 5190–200.

Maruyama, Y. 2002. Galpha12/13 Mediates alpha1-Adrenergic Receptor-Induced Cardiac Hypertrophy. Circulation Research, 91(10): 961–969.

McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., & Lefkowitz, R. J. 2000. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science (New York, N.Y.), 290(5496): 1574–7.

Mehta, P. B., Jenkins, B. L., McCarthy, L., Thilak, L., Robson, C. N., Neal, D. E., & Leung, H. Y. 2003. MEK5 overexpression is associated with metastatic prostate cancer, and stimulates proliferation, MMP-9 expression and invasion. Oncogene, 22(9): 1381–9.

Mellor, H., & Parker, P. J. 1998. The extended protein kinase C superfamily. The Biochemical journal, 332 (Pt 2: 281–92.

Mende, U., Semsarian, C., Martins, D. C., Kagen, a, Duffy, C., Schoen, F. J., & Neer, E. J. 2001. Dilated cardiomyopathy in two transgenic mouse lines expressing activated G protein alpha(q): lack of correlation between phospholipase C activation and the phenotype. Journal of molecular and cellular cardiology, 33(8): 1477–91.

Meshki, J., Douglas, S. D., Lai, J.-P., Schwartz, L., Kilpatrick, L. E., & Tuluc, F. 2009. Neurokinin 1 receptor mediates membrane blebbing in HEK293 cells through a Rho/Rho-associated coiled-coil kinase-dependent mechanism. The Journal of biological chemistry, 284(14): 9280–9.

Michnick, S., Ear, P. H., Landry, C., Malleshaiah, M. K., & Messier, V. 2011. Protein-Fragment Complementation Assays for Large-Scale Analysis, Functional Dissection and Dynamic Studies of Protein–Protein Interactions in Living Cells. (L. M. Luttrell & S. S. G. Ferguson, Eds.)Signal Transduction Protocols, 756: 395–425.

Milligan, G., & Kostenis, E. 2006. Heterotrimeric G-proteins: a short history. British journal of pharmacology, 147 Suppl : S46–55.

Mills, J. C., Stone, N. L., & Pittman, R. N. 1999. Extranuclear apoptosis. The role of the cytoplasm in the execution phase. The Journal of cell biology, 146(4): 703–8.

Minamino, T., Yujiri, T., Terada, N., Taffet, G. E., Michael, L. H., Johnson, G. L., & Schneider, M. D. 2002. MEKK1 is essential for cardiac hypertrophy and dysfunction induced by Gαq. Proceedings of the National Academy of Sciences of the United States of America, 99(6): 3866–71.

Minshall, R. D., Vandenbroucke, E. E., Holinstat, M., Place, A. T., Tiruppathi, C., Vogel, S. M., Van Nieuw Amerongen, G. P., Mehta, D., & Malik, A. B. 2010. Role of protein kinase Czeta in thrombin-induced RhoA activation and inter-endothelial gap formation of human dermal microvessel endothelial cell monolayers. Microvascular research, 80(2): 240–9.

Mishra, S., Ling, H., Grimm, M., Zhang, T., Bers, D. M., & Brown, J. H. 2010. Cardiac hypertrophy and heart failure development through Gαq and CaM kinase II signaling. Journal of cardiovascular pharmacology, 56(6): 598–603.

Miyata, M., Finch, E. A., Khiroug, L., Hashimoto, K., Hayasaka, S., Oda, S. I., Inouye, M., Takagishi, Y., Augustine, G. J., & Kano, M. 2000. Local calcium release in dendritic spines required for long-term synaptic depression. Neuron, 28(1): 233–44.

Mizuno, N., & Itoh, H. 2009. Functions and regulatory mechanisms of Gαq-signaling pathways. Neuro-Signals, 17(1): 42–54.

Mochly-Rosen, D., Wu, G., Hahn, H., Osinska, H., Liron, T., Lorenz, J. N., Yatani, A., Robbins, J., & Dorn, G. W. 2000. Cardiotrophic Effects of Protein Kinase C : Analysis by In Vivo Modulation of PLCεpsilon Translocation. Circulation Research, 86(11): 1173–1179.

Mohamed, A. J., Yu, L., Bäckesjö, C.-M., Vargas, L., Faryal, R., Aints, A., Christensson, B., Berglöf, A., Vihinen, M., Nore, B. F., & Smith, C. I. E. 2009. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. Immunological reviews, 228(1): 58–73.

Mollnau, H. 2002. Effects of Angiotensin II Infusion on the Expression and Function of NAD(P)H Oxidase and Components of Nitric Oxide/cGMP Signaling. Circulation Research, 90(4): 58e–65.

Moscat, J., & Diaz-Meco, M. T. 2000. The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. EMBO reports, 1(5): 399–403.

Moscat, J., & Diaz-Meco, M. T. 2009. P62 At the Crossroads of Autophagy, Apoptosis, and Cancer. Cell, 137(6): 1001–4.

Moscat, J., Diaz-Meco, M. T., & Wooten, M. W. 2009. Of the atypical PKCs, Par-4 and p62: recent understandings of the biology and pathology of a PB1-dominated complex. Cell death and differentiation, 16(11): 1426–37.

Moscat, J., Rennert, P., & Diaz-Meco, M. T. 2006. PKCζ at the crossroad of NF-kappaB and Jak1/Stat6 signaling pathways. Cell death and differentiation, 13(5): 702–11.

Mulloy, R., Salinas, S., Philips, A., & Hipskind, R. A. 2003. Activation of cyclin D1 expression by the ERK5 cascade. Oncogene, 22(35): 5387–98.

Münzel, F., Mühlhäuser, U., Zimmermann, W.-H., Didié, M., Schneiderbanger, K., Schubert, P., Engmann, S., Eschenhagen, T., & Zolk, O. 2005. Endothelin-1 and isoprenaline costimulation causes contractile failure which is partially reversed by MEK inhibition. Cardiovascular research, 68(3): 464–74.

Nakamura, K., & Johnson, G. L. 2003. PB1 domains of MEKK2 and MEKK3 interact with the MEK5 PB1 domain for activation of the ERK5 pathway. The Journal of biological chemistry, 278(39): 36989–92.

Nakamura, K., Uhlik, M., & Johnson, N. 2006. PB1 domain-dependent signaling complex is required for extracellular signal-regulated kinase 5 activation. Molecular and Cellular Biology, 26(6): 2065–2079.

Nebigil, C. G., Choi, D. S., Dierich, A., Hickel, P., Le Meur, M., Messaddeq, N., Launay, J. M., & Maroteaux, L. 2000. Serotonin 2B receptor is required for heart development. Proceedings of the National Academy of Sciences of the United States of America, 97(17): 9508–13.

Neves, S. R., Ram, P. T., & Iyengar, R. 2002. G protein pathways. Science (New York, N.Y.), 296(5573): 1636–9.

Newton, a C. 1995. Protein kinase C: structure, function, and regulation. The Journal of biological chemistry, 270(48): 28495–8.

Newton, A. C. 2003. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. The Biochemical journal, 370(Pt 2): 361–71.

Nicol, R. L., Frey, N., Pearson, G., Cobb, M., Richardson, J., & Olson, E. N. 2001. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. The EMBO journal, 20(11): 2757–67.

Nigro, P., Abe, J., Woo, C.-H., Satoh, K., McClain, C., O'Dell, M. R., Lee, H., Lim, J.-H., Li, J., Heo, K.-S., Fujiwara, K., & Berk, B. C. 2010. PKCζ decreases eNOS protein stability via inhibitory phosphorylation of ERK5. Blood, 116(11): 1971–9.

Nishizuka, Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 9(7): 484–96.

Nithianandarajah-Jones, G. N., Wilm, B., Goldring, C. E. P., Müller, J., & Cross, M. J. 2012. ERK5: Structure, regulation and function. Cellular signalling, 24(11): 2187–2196.

Noda, Y., Kohjima, M., Izaki, T., Ota, K., Yoshinaga, S., Inagaki, F., Ito, T., & Sumimoto, H. 2003. Molecular recognition in dimerization between PB1 domains. The Journal of biological chemistry, 278(44): 43516–24.

Noma, T., Lemaire, A., Naga Prasad, S. V, Barki-Harrington, L., Tilley, D. G., Chen, J., Le Corvoisier, P., Violin, J. D., Wei, H., Lefkowitz, R. J., & Rockman, H. A. 2007. Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. The Journal of clinical investigation, 117(9): 2445–58.

Obara, Y., & Nakahata, N. 2010. The signaling pathway leading to extracellular signal-regulated kinase 5 (ERK5) activation via G-proteins and ERK5-dependent neurotrophic effects. Molecular pharmacology, 77(1): 10–6.

Offermanns, S., Zhao, L. P., Gohla, A., Sarosi, I., Simon, M. I., & Wilkie, T. M. 1998. Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. The EMBO journal, 17(15): 4304–12.

Oh, J. G., Jeong, D., Cha, H., Kim, J. M., Lifirsu, E., Kim, J., Yang, D. K., Park, C. S., Kho, C., Park, S., Yoo, Y. J., Kim, D. H., Kim, J., Hajjar, R. J., & Park, W. J. 2012. PICOT increases cardiac contractility by inhibiting PKCζ activity. Journal of molecular and cellular cardiology, 53(1): 53–63.

Oka, Y., Saraiva, L. R., Kwan, Y. Y., & Korsching, S. I. 2009. The fifth class of Galpha proteins. Proceedings of the National Academy of Sciences of the United States of America, 106(5): 1484–9.

Oldham, W. M., & E. Hamm, H. 2006. Structural basis of function in heterotrimeric G proteins. Quarterly Reviews of Biophysics, 39(02): 117–166.

Oldham, W. M., & Hamm, H. E. 2008. Heterotrimeric G protein activation by G-protein-coupled receptors. Nature reviews. Molecular cell biology, 9(1): 60–71.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., & Cobb, M. H. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine reviews, 22(2): 153–83.

Peavy, R. D., Chang, M. S., Sanders-Bush, E., & Conn, P. J. 2001. Metabotropic glutamate receptor 5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor. The Journal of neuroscience : the official journal of the Society for Neuroscience, 21(24): 9619–28.

Peavy, R., Hubbard, K., & Lau, A. 2005. Differential Effects of Gαq {alpha}, G14 {alpha}, and G15 {alpha} on Vascular Smooth Muscle Cell Survival and Gene Expression Profiles. Molecular pharmacology, 67(6): 2102–2114.

Pegtel, D. M., Ellenbroek, S. I. J., Mertens, A. E. E., Van der Kammen, R. a, De Rooij, J., & Collard, J. G. 2007. The Par-Tiam1 complex controls persistent migration by stabilizing microtubule-dependent front-rear polarity. Current biology : CB, 17(19): 1623–34.

Penela, P., Murga, C., Ribas, C., Lafarga, V., & Mayor, F. 2010. The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. British journal of pharmacology, 160(4): 821–32.

Penela, P., Murga, C., Ribas, C., Tutor, A. S., Peregrín, S., & Mayor, F. 2006. Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. Cardiovascular research, 69(1): 46–56.

Pesanová, Z., Novotný, J., Cerný, J., Milligan, G., & Svoboda, P. 1999. Thyrotropin-releasing hormone-induced depletion of G(q)alpha/G(11)alpha proteins from detergent-insensitive membrane domains. FEBS letters, 464(1-2): 35–40.

Phillipson, A., Peterman, E. E., Taormina, P., Harvey, M., Brue, R. J., Atkinson, N., Omiyi, D., Chukwu, U., & Young, L. H. 2005. Protein kinase C-zeta inhibition exerts cardioprotective effects in ischemia-reperfusion injury. American journal of physiology. Heart and circulatory physiology, 289(2): H898–907.

Pi, X., Yan, C., & Berk, B. C. 2004. Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. Circulation research, 94(3): 362–9.

Pierce, K. L., Premont, R. T., & Lefkowitz, R. J. 2002. Seven-transmembrane receptors. Nature reviews. Molecular cell biology, 3(9): 639–50.

Pin, J.-P., Neubig, R., Bouvier, M., Devi, L., Filizola, M., Javitch, J. A., Lohse, M. J., Milligan, G., Palczewski, K., Parmentier, M., & Spedding, M. 2007. International Union of Basic and Clinical Pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. Pharmacological reviews, 59(1): 5–13.

Popova, J. S., & Rasenick, M. M. 2003. G beta gamma mediates the interplay between tubulin dimers and microtubules in the modulation of Gαq signaling. The Journal of biological chemistry, 278(36): 34299–308.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., & Ullrich, A. in press. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature, 402(6764): 884–8. doi:10.1038/47260

Puls, A., Schmidt, S., Grwe, F., & Stabel, S. 1997. Interaction of protein kinase C z with ZIP , a novel protein kinase C-binding protein. Proceedings of the National Academy of Sciences, 94(June): 6191–6196.

Qiu, Y., Wang, Y., Law, P., Chen, H., & Loh, H. H. 2011. Cholesterol Regulates -Opioid Receptor-Induced β-Arrestin 2 Translocation to Membrane Lipid Rafts. Molecular Pharmacology, 210–218.

Raamsdonk, C. Van, Bezrookove, V., & Green, G. 2008. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature, 457(January).

Rajagopal, S., Rajagopal, K., & Lefkowitz, R. J. 2010. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nature reviews. Drug discovery, 9(5): 373–86.

Rakesh, K., Yoo, B., Kim, I.-M., Salazar, N., Kim, K.-S., & Rockman, H. a. 2010. beta-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. Science signaling, 3(125): ra46.

Rall, T. W., & Sutherland, E. W. 1958. Formation of a cyclic adenine ribonucleotide by tissue particles. The Journal of biological chemistry, 232(2): 1065–76.

Rapacciuolo, A., Esposito, G., Prasad, S. V, & Rockman, H. A. 2001. G protein-coupled receptor signalling in in vivo cardiac overload. Acta physiologica Scandinavica, 173(1): 51–7.

Rasmussen, S. G. F., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T. a., Lyons, J. a., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., & Kobilka, B. K. 2011. Crystal structure of the β2 adrenergic receptor–Gs protein complex. Nature, 477(7366): 549–555.

Raviv, Z., Kalie, E., & Seger, R. 2004. MEK5 and ERK5 are localized in the nuclei of resting as well as stimulated cells, while MEKK2 translocates from the cytosol to the nucleus upon stimulation. Journal of cell science, 117(Pt 9): 1773–84.

Reina, S., Sterin-Borda, L., Passafaro, D., & Borda, E. 2010. Muscarinic cholinoceptor activation by pilocarpine triggers apoptosis in human skin fibroblast cells. Journal of cellular physiology, 222(3): 640–7.

Resende, R. R., & Adhikari, A. 2009. Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. Cell communication and signaling : CCS, 7: 20.

Rhee, S. 2001. Regulation of phosphoinositide-specific phospholipase C*. Annual review of biochemistry, 70: 281–312.

Ribas, C., Penela, P., Murga, C., Salcedo, A., García-Hoz, C., Jurado-Pueyo, M., Aymerich, I., & Mayor, F. 2007. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. Biochimica et biophysica acta, 1768(4): 913–22.

Ridley, A. J. 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends in cell biology, 16(10): 522–9.

Roberts, O. L., Holmes, K., Müller, J., Cross, D. a E., & Cross, M. J. 2009. ERK5 and the regulation of endothelial cell function. Biochemical Society transactions, 37(Pt 6): 1254–9.

Rochdi, M. D., & Parent, J.-L. 2003. Galphaq-coupled receptor internalization specifically induced by Galphaq signaling. Regulation by EBP50. The Journal of biological chemistry, 278(20): 17827–37.

Rochdi, M. D., Watier, V., La Madeleine, C., Nakata, H., Kozasa, T., & Parent, J.-L. 2002. Regulation of GTP-binding protein alpha q (Galpha q) signaling by the ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50). The Journal of biological chemistry, 277(43): 40751–9.

Rockman, H. A., Choi, D. J., Rahman, N. U., Akhter, S. A., Lefkowitz, R. J., & Koch, W. J. 1996. Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America, 93(18): 9954–9.

Rodbell, M., Krans, H. M. J., Pohl, S. L., & Birnbaumer, L. 1971. The Glucagon-sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver. III. BINDING OF GLUCAGON: METHOD OF ASSAY AND SPECIFICITY. J. Biol. Chem., 246(6): 1861–1871.

Rogers, J. H., Tamirisa, P., Kovacs, A., Weinheimer, C., Courtois, M., Blumer, K. J., Kelly, D. P., & Muslin, A. J. 1999. RGS4 causes increased mortality and reduced cardiac hypertrophy in response to pressure overload. The Journal of clinical investigation, 104(5): 567–76.

Rohini, A., Agrawal, N., Koyani, C. N., & Singh, R. 2010. Molecular targets and regulators of cardiac hypertrophy. Pharmacological research : the official journal of the Italian Pharmacological Society, 61(4): 269–80.

Rojas, R. J., Yohe, M. E., Gershburg, S., Kawano, T., Kozasa, T., & Sondek, J. 2007. Galphaq directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain. The Journal of biological chemistry, 282(40): 29201–10.

Rose, B. A., Force, T., & Wang, Y. 2010. Mitogen-Activated Protein Kinase Signaling in the Heart : Angels Versus Demons in a Heart-Breaking Tale. Physiological Reviews, 90(4): 1507–1546.

Ross, E. M. 2011. G{alpha}q and Phospholipase C-{beta}: Turn On, Turn Off, and Do It Fast. Science signaling, 4(159): pe5.

Ross, E. M., & Gilman, A. G. 1977. Resolution of some components of adenylate cyclase necessary for catalytic activity. The Journal of biological chemistry, 252(20): 6966–9.

Rossman, K. L., Der, C. J., & Sondek, J. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nature reviews. Molecular cell biology, 6(2): 167–80.

Sagi, S. A., Seasholtz, T. M., Kobiashvili, M., Wilson, B. A., Toksoz, D., & Brown, J. H. 2001. Physical and functional interactions of Galphaq with Rho and its exchange factors. The Journal of biological chemistry, 276(18): 15445–52.

Salles, G., Leocádio, S., Bloch, K., Nogueira, A. R., & Muxfeldt, E. 2005. Combined QT interval and voltage criteria improve left ventricular hypertrophy detection in resistant hypertension. Hypertension, 46(5): 1207–12.

Sallese, M., Mariggiò, S., D'Urbano, E., Iacovelli, L., & De Blasi, A. 2000. Selective regulation of Gαq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq. Molecular pharmacology, 57(4): 826–31.

Samuels, I. S., Seibenhener, M. L., Neidigh, K. B., & Wooten, M. W. 2001. Nerve growth factor stimulates the interaction of ZIP/p62 with atypical protein kinase C and targets endosomal localization: evidence for regulation of nerve growth factor-induced differentiation. Journal of cellular biochemistry, 82(3): 452–66.

Sanz, L., Sanchez, P., Lallena, M. J., Diaz-Meco, M. T., & Moscat, J. 1999. The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation. The EMBO journal, 18(11): 3044–53.

Sarma, T., Voyno-Yasenetskaya, T., Hope, T. J., & Rasenick, M. M. 2003. Heterotrimeric G-proteins associate with microtubules during differentiation in PC12 pheochromocytoma cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 17(8): 848–59.

Sengupta, P., Philip, F., & Scarlata, S. 2008. Caveolin-1 alters Ca(2+) signal duration through specific interaction with the G alpha q family of G proteins. Journal of cell science, 121(Pt 9): 1363–72.

Seo, B., Choy, E. W., Maudsley, S., Miller, W. E., Wilson, B. a, & Luttrell, L. M. 2000. Pasteurella multocida toxin stimulates mitogen-activated protein kinase via G(q/11)-dependent transactivation of the epidermal growth factor receptor. The Journal of biological chemistry, 275(3): 2239–45.

Shafer, S. H., & Williams, C. L. 2004. Elevated Rac1 activity changes the M3 muscarinic acetylcholine receptor-mediated inhibition of proliferation to induction of cell death. Molecular pharmacology, 65(5): 1080–91.

Shankaranarayanan, A., Boguth, C. A., Lutz, S., Vettel, C., Uhlemann, F., Aittaleb, M., Wieland, T., & Tesmer, J. J. G. 2010. Galpha q allosterically activates and relieves autoinhibition of p63RhoGEF. Cellular signalling, 22(7): 1114–23.

Shankaranarayanan, A., Thal, D. M., Tesmer, V. M., Roman, D. L., Neubig, R. R., Kozasa, T., & Tesmer, J. J. G. 2008. Assembly of high order G alpha q-effector complexes with RGS proteins. The Journal of biological chemistry, 283(50): 34923–34.

Shenoy, S. K., McDonald, P. H., Kohout, T. A., & Lefkowitz, R. J. 2001. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science (New York, N.Y.), 294(5545): 1307–13.

Shizukuda, Y., & Buttrick, P. M. 2002. Protein kinase C-zeta modulates thromboxane A(2)-mediated apoptosis in adult ventricular myocytes via Akt. American journal of physiology. Heart and circulatory physiology, 282(1): H320–7.

Simon, M., Strathmann, M., & Gautam, N. 1991. Diversity of G proteins in signal transduction. Science, 252(5007): 802–808.

Simons, K., & Toomre, D. 2000. Lipid rafts and signal transduction. Nature reviews. Molecular cell biology, 1(1): 31–9.

Smith, L., Chen, L., Reyland, M. E., DeVries, T. a, Talanian, R. V, Omura, S., & Smith, J. B. 2000. Activation of atypical protein kinase C zeta by caspase processing and degradation by the ubiquitin-proteasome system. The Journal of biological chemistry, 275(51): 40620–7.

Smith, L., & Smith, J. B. 2002. Lack of constitutive activity of the free kinase domain of protein kinase C zeta. Dependence on transphosphorylation of the activation loop. The Journal of biological chemistry, 277(48): 45866–73.

Smith, L., Wang, Z., & Smith, J. B. 2003. Caspase processing activates atypical protein kinase C zeta by relieving autoinhibition and destabilizes the protein. The Biochemical journal, 375(Pt 3): 663–71.

Snyder, S. H., & Pasternak, G. W. 2003. Historical review: Opioid receptors. Trends in pharmacological sciences, 24(4): 198–205.

Sohn, S. J., Lewis, G. M., & Winoto, A. 2008. Non-redundant function of the MEK5-ERK5 pathway in thymocyte apoptosis. The EMBO journal, 27(13): 1896–906.

Soloff, R. S., Katayama, C., Lin, M. Y., Feramisco, J. R., & Hedrick, S. M. 2004. Targeted deletion of protein kinase C lambda reveals a distribution of functions between the two atypical protein kinase C isoforms. Journal of immunology (Baltimore, Md. : 1950), 173(5): 3250–60.

Sorkin, A., & Von Zastrow, M. 2009. Endocytosis and signalling: intertwining molecular networks. Nature reviews. Molecular cell biology, 10(9): 609–22.

Sprang, S. R., Chen, Z., & Du, X. 2007. Structural basis of effector regulation and signal termination in heterotrimeric Galpha proteins. Advances in protein chemistry, 74(07): 1–65.

Spruill, L. S., & McDermott, P. J. 2006. Regulation of c-jun mRNA expression in adult cardiocytes by MAP kinase interacting kinase-1 (MNK1). FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 20(12): 2133–5.

Squier, M. K., & Cohen, J. J. 2001. Standard quantitative assays for apoptosis. Molecular biotechnology, 19(3): 305–12.

Standaert, M., Bandyopadhyay, G., & Kanoh, Y. 2001. Insulin and PIP3 Activate PKC-zeta by Mechanisms That Are Both Dependent and Independent of Phosphorylation of Activation Loop (T410) and Autophosphorylation (T560) Sites. Biochemistry, 40(1): 249–255.

Standfuss, J., Edwards, P. C., D'Antona, A., Fransen, M., Xie, G., Oprian, D. D., & Schertler, G. F. X. 2011. The structural basis of agonist-induced activation in constitutively active rhodopsin. Nature, 471(7340): 656–60.

Stanislaus, D., Janovick, J. A., Brothers, S., & Conn, P. M. 1997. Regulation of G(q/11) alpha by the gonadotropin-releasing hormone receptor. Molecular endocrinology (Baltimore, Md.), 11(6): 738–46.

Stefan, E., Aquin, S., Berger, N., Landry, C. R., Nyfeler, B., Bouvier, M., & Michnick, S. W. 2007. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. Proceedings of the National Academy of Sciences of the United States of America, 104(43): 16916–21.

Stefan, E., Malleshaiah, M. K., Breton, B., Ear, P. H., Bachmann, V., Beyermann, M., Bouvier, M., & Michnick, S. W. 2011. PKA regulatory subunits mediate synergy among conserved G-protein-coupled receptor cascades. Nature communications, 2: 598.

Sterne-Marr, R., Tesmer, J. J. G., Day, P. W., Stracquatanio, R. P., Cilente, J.-A. E., O'Connor, K. E., Pronin, A. N., Benovic, J. L., & Wedegaertner, P. B. 2003. G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signaling homology domain for binding G alpha subunits. The Journal of biological chemistry, 278(8): 6050–8.

Strassheim, D., May, L. G., Varker, K. a, Puhl, H. L., Phelps, S. H., Porter, R. a, Aronstam, R. S., Noti, J. D., & Williams, C. L. 1999. M3 muscarinic acetylcholine receptors regulate cytoplasmic myosin by a process involving RhoA and requiring conventional protein kinase C isoforms. The Journal of biological chemistry, 274(26): 18675–85.

Street, M., Marsh, S. J., Stabach, P. R., Morrow, J. S., Brown, D. a, & Buckley, N. J. 2006. Stimulation of Galphaq-coupled M1 muscarinic receptor causes reversible spectrin redistribution mediated by PLC, PKC and ROCK. Journal of cell science, 119(Pt 8): 1528–36.

Sugawara, Y., Nishii, H., Takahashi, T., Yamauchi, J., Mizuno, N., Tago, K., & Itoh, H. 2007. The lipid raft proteins flotillins/reggies interact with Galphaq and are involved in Gαq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cellular signalling, 19(6): 1301–8.

Sumimoto, H., Kamakura, S., & Ito, T. 2007. Structure and function of the PB1 domain, a protein interaction module conserved in animals, fungi, amoebas, and plants. Science's STKE : signal transduction knowledge environment, 2007(401): re6.

Swenson-Fields, K. I., Sandquist, J. C., Rossol-Allison, J., Blat, I. C., Wennerberg, K., Burridge, K., & Means, A. R. 2008. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Molecular cell, 32(1): 43–56.

Takeda, N., Manabe, I., Uchino, Y., Eguchi, K., Matsumoto, S., Nishimura, S., Shindo, T., Sano, M., Otsu, K., Snider, P., Conway, S. J., & Nagai, R. 2010. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload, 120(1).

Takeishi, Y., Huang, Q., Abe, J., Glassman, M., Che, W., Lee, J. D., Kawakatsu, H., Lawrence, E. G., Hoit, B. D., Berk, B. C., & Walsh, R. A. 2001. Src and multiple MAP kinase activation in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch. Journal of molecular and cellular cardiology, 33(9): 1637–48.

Tesmer, J. J., Berman, D. M., Gilman, a G., & Sprang, S. R. 1997. Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell, 89(2): 251–61.

Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., & Tesmer, J. J. G. 2005. Snapshot of activated G proteins at the membrane: the Galphaq-GRK2-Gbetagamma complex. Science (New York, N.Y.), 310(5754): 1686–90.

Tilley, D. G. 2011. G protein-dependent and g protein-independent signaling pathways and their impact on cardiac function. Circulation research, 109(2): 217–30.

Tobin, a B., & Budd, D. C. 2003. The anti-apoptotic response of the Gαq/11-coupled muscarinic receptor family. Biochemical Society transactions, 31(Pt 6): 1182–5.

Torrecilla, I., Spragg, E. J., Poulin, B., McWilliams, P. J., Mistry, S. C., Blaukat, A., & Tobin, A. B. 2007. Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. The Journal of cell biology, 177(1): 127–37.

Tsuboi, K., Kawano, T., & Kozasa, T. 2008. Identification of Gαq mutants specifically uncoupled with PLCβ and GRK2. 3rd RGS Protein Colloquium.

Turjanski, A. G., Vaqué, J. P., & Gutkind, J. S. 2007. MAP kinases and the control of nuclear events. Oncogene, 26(22): 3240–53.

Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K., & Yamamoto, T. 1997. Activation of the G protein Gαq/11 through tyrosine phosphorylation of the alpha subunit. Science (New York, N.Y.), 276(5320): 1878–81.

Ungerer, M., Parruti, G., Bohm, M., Puzicha, M., DeBlasi, a., Erdmann, E., & Lohse, M. J. 1994. Expression of beta-arrestins and beta-adrenergic receptor kinases in the failing human heart. Circulation Research, 74(2): 206–213.

Usui, I., Imamura, T., Satoh, H., Huang, J., Babendure, J. L., Hupfeld, C. J., & Olefsky, J. M. 2004. GRK2 is an endogenous protein inhibitor of the insulin signaling pathway for glucose transport stimulation. The EMBO journal, 23(14): 2821–9.

Van Raamsdonk, C. D., Griewank, K., Crosby, M. B., Garrido, M. C., Vemula, S., Wiesner, T., Obenauf, A. C., Wackernagel, W., Green, G., Bouvier, N., & others. 2010. Mutations in GNA11 in uveal melanoma. New England Journal of Medicine, 363(23): 2191–2199.

Venkatakrishnan, G., & Exton, J. H. 1996. Identification of determinants in the alpha-subunit of Gαq required for phospholipase C activation. The Journal of biological chemistry, 271(9): 5066–72.

Violin, J. D., DeWire, S. M., Yamashita, D., Rominger, D. H., Nguyen, L., Schiller, K., Whalen, E. J., Gowen, M., & Lark, M. W. 2010. Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. The Journal of pharmacology and experimental therapeutics, 335(3): 572–9.

Vogt, S., Grosse, R., Schultz, G., & Offermanns, S. 2003. Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gαq/Gα11. The Journal of biological chemistry, 278(31): 28743–9.

Waldo, G. L., Ricks, T. K., Hicks, S. N., Cheever, M. L., Kawano, T., Tsuboi, K., Wang, X., Montell, C., Kozasa, T., Sondek, J., & Harden, T. K. 2010a. Kinetic Scaffolding Mediated by a Phospholipase C-{beta} and Gαq Signaling Complex. Science (New York, N.Y.), 330(6006): 974–80.

Waldo, G. L., Ricks, T. K., Hicks, S. N., Cheever, M. L., Kawano, T., Tsuboi, K., Wang, X., Montell, C., Kozasa, T., Sondek, J., & Harden, T. K. 2010b. Kinetic scaffolding mediated by a phospholipase C-beta and Gαq signaling complex. Science (New York, N.Y.), 330(6006): 974–80.

Walsh, D. A., Perkins, J. P., & Krebs, E. G. 1968. An Adenosine 3',5'-Monophosphate-dependant Protein Kinase from Rabbit Skeletal Muscle. J. Biol. Chem., 243(13): 3763–3765.

Wang, N., Yan, K., & Rasenick, M. M. 1990. Tubulin binds specifically to the signal-transducing proteins, Gs alpha and Gi alpha 1. The Journal of biological chemistry, 265(3): 1239–42.

Wang, X., & Tournier, C. 2006. Regulation of cellular functions by the ERK5 signalling pathway. Cellular signalling, 18(6): 753–60.

Wang, Y., Huang, S., Sah, V. P., Ross, J., Brown, J. H., Han, J., & Chien, K. R. 1998a. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. The Journal of biological chemistry, 273(4): 2161–8.

Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., & Chien, K. R. 1998b. Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. The Journal of biological chemistry, 273(10): 5423–6.

Watanabe, T., Barker, T. a, & Berk, B. C. 2005. Angiotensin II and the endothelium: diverse signals and effects. Hypertension, 45(2): 163–9.

Watson, F. L., Heerssen, H. M., Bhattacharyya, a, Klesse, L., Lin, M. Z., & Segal, R. a. 2001. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nature neuroscience, 4(10): 981–8.

Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., & Blumer, K. J. 1996. RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature, 383(6596): 172–5.

Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., & Bourne, H. R. 1993. Palmitoylation is required for signaling functions and membrane attachment of Gαq alpha and Gs alpha. The Journal of biological chemistry, 268(33): 25001–8.

Welch, H. C. E., Coadwell, W. J., Ellson, C. D., Ferguson, G. J., Andrews, S. R., Erdjument-Bromage, H., Tempst, P., Hawkins, P. T., & Stephens, L. R. 2002. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell, 108(6): 809–21.

Wettschureck, N., & Offermanns, S. 2005. Mammalian G Proteins and Their Cell Type Specific Functions. Physiological Reviews, 1159–1204.

Wettschureck, N., Rütten, H., Zywietz, A., Gehring, D., Wilkie, T. M., Chen, J., Chien, K. R., & Offermanns, S. 2001. Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. Nature medicine, 7(11): 1236–40.

Widmann, C., Gibson, S., Jarpe, M. B., & Johnson, G. L. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiological reviews, 79(1): 143–80.

Wilden, U. 1995. Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding. Biochemistry, 34(4): 1446–54.

Williams, S. L., Lutz, S., Charlie, N. K., Vettel, C., Ailion, M., Coco, C., Tesmer, J. J. G., Jorgensen, E. M., Wieland, T., & Miller, K. G. 2007. Trio's Rho-specific GEF domain is the missing Galpha q effector in C. elegans. Genes & development, 21(21): 2731–46.

Wilson, M., Gill, D., Perisic, O., Quinn, M., & Williams, R. 2003. PB1 domain-mediated heterodimerization in NADPH oxidase and signaling complexes of atypical protein kinase C with Par6 and p62. Molecular cell, 12: 39–50.

Wise, A., Carr, I. C., Groarke, D. A., & Milligan, G. 1997. Measurement of agonist efficacy using an alpha2A-adrenoceptor-Gi1alpha fusion protein. FEBS letters, 419(1): 141–6.

Wooten, M. W. 1999. Function for NF-kB in neuronal survival: regulation by atypical protein kinase C. Journal of neuroscience research, 58(5): 607–11.

Wu, G., Toyokawa, T., Hahn, H., & Dorn, G. W. 2000. Epsilon protein kinase C in pathological myocardial hypertrophy. Analysis by combined transgenic expression of translocation modifiers and Galphaq. The Journal of biological chemistry, 275(39): 29927–30.

Wu, S. C., & Solaro, R. J. 2007. Protein kinase C zeta. A novel regulator of both phosphorylation and de-phosphorylation of cardiac sarcomeric proteins. The Journal of biological chemistry, 282(42): 30691–8.

Wu, X., Li, S., Chrostek-Grashoff, A., Czuchra, A., Meyer, H., Yurchenco, P. D., & Brakebusch, C. 2007. Cdc42 is crucial for the establishment of epithelial polarity during early mammalian development. Developmental dynamics : an official publication of the American Association of Anatomists, 236(10): 2767–78.

Xie, P., Browning, D. D., Hay, N., Mackman, N., & Ye, R. D. 2000. Activation of NF-kappa B by bradykinin through a Galpha(q)- and Gbeta gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt. The Journal of biological chemistry, 275(32): 24907–14.

Xu, J., Gong, N. L., Bodi, I., Aronow, B. J., Backx, P. H., & Molkentin, J. D. 2006. Myocyte enhancer factors 2A and 2C induce dilated cardiomyopathy in transgenic mice. The Journal of biological chemistry, 281(14): 9152–62.

Xu, X., Zeng, W., Popov, S., Berman, D. M., Davignon, I., Yu, K., Yowe, D., Offermanns, S., Muallem, S., & Wilkie, T. M. 1999. RGS proteins determine signaling specificity of Gq-coupled receptors. The Journal of biological chemistry, 274(6): 3549–56.

Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., De Wit, D., Emoto, N., & Hammer, R. E. 1998. Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. Development (Cambridge, England), 125(5): 825–36.

Yang, J. M., Cho, C. H., Kong, K. A., Jang, I. S., Kim, H. W., & Juhnn, Y. S. 1999. Increased expression of Galphaq protein in the heart of streptozotocin-induced diabetic rats. Experimental & molecular medicine, 31(4): 179–84.

Yu, J.-Z., & Rasenick, M. M. 2002. Real-time visualization of a fluorescent G(alpha)(s): dissociation of the activated G protein from plasma membrane. Molecular pharmacology, 61(2): 352–9.

Zhan, Y., Brown, C., Maynard, E., Anshelevich, A., Ni, W., Ho, I.-C., & Oettgen, P. 2005. Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling. The Journal of clinical investigation, 115(9): 2508–16.

Zimmerman, B., Simaan, M., Lee, M.-H., Luttrell, L. M., & Laporte, S. A. 2009. c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway. Cellular signalling, 21(1): 103–10.

Zimmerman, M. C., & Zucker, I. H. 2009. Mitochondrial dysfunction and mitochondrial-produced reactive oxygen species: new targets for neurogenic hypertension? Hypertension, 53(2): 112–4.

VIII. RESUMEN EN CASTELLANO

INTRODUCCIÓN

Los receptores acoplados a proteínas G (GPCR) son la familia más numerosa de receptores de membrana en mamíferos y tienen un papel esencial en procesos fisiopatológicos. La señalización por GPCRs depende de la activación de proteínas G y, de manera alternativa, del reclutamiento de β-arrestinas. La proteína Gαq es un importante mediador intracelular de la propagación de la señal iniciada tras la estimulación de GPCRs. Su función está regulada por la quinasa de receptores GRK2 y por las proteínas RGS. Gαq interacciona con más de 20 proteínas celulares, denominadas efectores, a través de las cuales inicia diversas cascadas de señalización. Por tanto, la funcionalidad de Gαq depende directamente de los efectores celulares a los que active. El ejemplo más paradigmático de proteína efectora de Gαq es la enzima PLCβ, encargada de la conversión de PIP2 en IP3 y diacilglicerol. Estos dos segundos mensajeros inician diversas vías intracelulares dependientes de lípidos y calcio. Sin embargo, recientemente se han descubierto numerosas cascadas de señalización dependientes de Gαq que ocurren al margen de PLCβ. Por ejemplo, la familia de proteínas RhoGEF (del inglés Rho guanine exchange factors) se unen a Gαq lo cual promueve la activación de la proteína Rho por GPCR. Recientemente, hemos descrito una nueva activación de la vía de ERK5 por GPCR que depende de la interacción de Gαq con dos nuevos efectores, PKCζ y MEK5. Esta vía es el objeto de estudio del presente trabajo y la introducción se ha centrado en describir procesos relevantes en dicho contexto de señalización.

Las proteínas quinasas C (PKC) son una gran familia de serina/treonina quinasas con importantes roles en señalización celular. PKCζ pertenece a la familia de las PKC atípicas ya que, al contrario que las PKC clásicas, no pueden ser activadas por calcio ni diacilglicerol Sin embargo, estas quinasas atípicas muestran una interesante variedad de funciones a través de interacciones proteína-proteína. Tanto PKCζ como PLCλ (el otro miembro de las PKC atípicas) tienen un dominio PB1 a través del cual interaccionan con diversas proteínas, participando de esta manera en procesos como la polaridad celular, la activación de la vía NFκB o la respuesta alérgica y el asma.

Las quinasas activadas por mitógenos (MAPKs) son serina/treonina quinasas con una función esencial en el control de la transcripción en respuesta a diversos estímulos externos. ERK5 es el miembro más reciente de esta gran familia y, por lo tanto, el más desconocido. Esta proteína es fosforilada dualmente en su dominio quinasa, como el resto de las MAPK, pero presenta de manera diferencial un singular dominio C-terminal a través del cual es capaz de actuar de activador transcripcional. ERK5 es una proteína que promueve la supervivencia y juega un papel esencial en el desarrollo cardiaco, a través de su función endotelial.

Gαq es un importante mediador de la función cardiaca. Su sobreexpresión está asociada con hipertrofia cardiaca y fallo cardiaco. Hemos descrito que la vía de activación de ERK5 a través de PKCζ tiene un papel fundamental en el desarrollo de hipertrofia en respuesta a la estimulación crónica con Angiotensina II. Por tanto, el estudio detallado de los mecanismos bioquímicos y celulares de esta nueva vía puede aportar datos relevantes para el tratamiento de patologías cardiacas.

OBJETIVOS

1. Determinar si la vía de señalización Gαq/PKCζ/ERK5 es operativa en tejido cardiaco.

2. Determinar la contribución relativa de Gαq y β-arrestinas en la activación de ERK5 por GPCRs acoplados a Gαq.

3. Determinar la especificidad del complejo Gαq/PKCζ en células vivas.

4. Identificar las superficies de interacción entre Gαq y PKCζ .

5. Determinar la dependencia de la vía de ERK5 por el complejo Gαq/PKCζ.

6. Estudiar la función reguladora de GRK2 en la vía de activación de ERK5 por GPCRs acoplados a Gαq.

7. Estudiar los mecanismos de activación del nuevo efector PKCζ en la vía de activación de ERK5 por GPCRs acoplados a Gαq.

8. Explorar las funciones potenciales del complejo Gαq/PKCζ en procesos celulares dependientes de la activación de Gαq.

RESULTADOS

La célula es una entidad dinámica compuesta de redes bioquímicas altamente intrincadas que le permiten responder de una manera orquestada a distintas perturbaciones. Las proteínas son elementos fundamentales de dichas redes y forman circuitos de alta complejidad a través de interacciones proteína-proteína. Las proteínas G son componentes celulares esenciales para la señalización de los receptores acoplados a proteínas G (GPCR). Esta función se ejerce a través de interacciones específicas, y conservadas a lo largo de la evolución, entre proteínas G activadas y numerosos efectores celulares. Recientemente hemos descrito que la activación de ERK5 por GPCRs acoplados a Gαq precisa de la participación de un nuevo efector, PKCζ. En este estudio presentamos una caracterización bioquímica y celular del eje de señalización Gαq/PKCζ. Hemos demostrado que la cascada Gαq/PKCζ es responsable de la activación de ERK5 en respuesta a Angiotensina II en el corazón. También hemos descrito que esta cascada de señalización tiene propiedades sesgadas hacia Gαq, lo cual puede tener implicaciones terapéuticas interesantes. El complejo Gαq/PKCζ ha sido caracterizado en células in situ. Además, mediante experimentos de mutagénesis se han identificado residuos importantes en ambas proteínas para esta interacción. Así, la lisina 19, localizada en el dominio PB1 de PKCζ, y los ácidos glutámicos en posiciones 234 y 245, localizados en una nueva región de Gαq denominada dominio pseudo-PB1, parecen ser aminoácidos esenciales para la formación del complejo Gαq/PKCζ. Notablemente, la doble mutación (E234/E245-AA) en Gαq impide la activación de ERK5, demostrando el requerimiento de un complejo Gαq/PKCζ eficiente para la activación de la cascada. También hemos descrito que GRK2, pero no RGS2/4, previene la asociación de PKCζ con Gαq, mediante el secuestro de la proteína Gαq. Este efecto de GRK2, junto con la desensibilización de receptores también promovida por GRK2, resulta en la inhibición de la activación de ERK5 por GPCRs acoplados a Gαq. Además, hemos descrito que PKCζ no es activado por GPCRs a través de fosforilación y demostramos, por primera vez, la dimerización transitoria de PKCζ inducida por un receptor. Esta proteína actúa de andamio entre la proteína Gαq activada y ERK5. Finalmente, hemos estudiado la participación del complejo Gαq/PKCζ en las funciones celulares de Gαq . La formación de este complejo demostró ser necesaria para el “blebbing” de membrana y la parada del crecimiento celular inducidos por el receptor muscarínico M3. Nuestros resultados también apuntan a la participación del complejo Gαq/PKCζ en la promoción de apoptosis en situaciones de privación de nutrientes. Globalmente, este estudio aporta datos bioquímicos y celulares de gran relevancia para entender la señalización de GPCRs acoplados a Gαq a través del nuevo efector PKCζ.

DISCUSIÓN

Hemos descrito la vía de activación de ERK5 por GPCR acoplados a Gαq en cardiomiocitos, fibroblastos y tejido cardiaco. Estos resultados apoyan la conservación de la vía previamente descrita en células epiteliales y sugiere importantes funciones para este nuevo eje de señalización. Así, PKCζ se confirma como el efector fundamental de Gαq en la activación de ERK5. Como parte de otra tesis se describió la implicación de esta vía en el desarrollo de hipertrofia cardiaca. En este trabajo, hemos especulado sobre los posibles mecanismos de este fenómeno proponiendo un modelo en el que ERK5 es responsable de la secreción de colágeno en fibroblastos, el cual se uniría a integrinas en la superficie de los cardiomiocitos, promoviendo hipertrofia de manera aditiva a la propia activación de ERK5 en este tipo celular. En el contexto cardiovascular, resulta muy relevante, el haber determinado la preponderancia de vías Gαq frente a vías β-arrestina, para la activación de ERK5 por GPCRs. Esto sugiere que la activación de ERK5 en corazón es estrictamente deletérea, al contrario que ERK1/2 que también participa en vías cardioprotectoras, y por tanto, podría constituir una diana terapéutica de interés para el tratamiento de la hipertrofia cardiaca.

La visualización del complejo PKCζ/Gαq en células vivas, de manera altamente específica, indica la relevancia celular de esta interacción. Sin embargo, la localización concreta del complejo no se ha podido determinar con seguridad, ya que en nuestro sistema se encuentra disperso por el citoplasma. Existe la posibilidad de que la adición de un epítopo a la proteína G haya interferido con su localización en la membrana. El reciente desarrollo de epítopos internos para proteínas G será de utilidad en futuros proyectos para clarificar este tema. El resultado más sorprendente en lo que se refiere a la especificidad del complejo es la ausencia de interacción entre PKCζ y Gα11, miembro de la familia Gαq/11 y con alta homología de secuencia con Gαq. El análisis de este resultado junto a los datos de las superficies de interacción del complejo Gαq/PKCζ presentados en esta tesis, sugiere que la deficiencia de asociación entre PKCζ y Gα11 no se debe a una diferencia en las interfases (idénticas en Gαq y Gα11), sino posiblemente a una diferente localización celular. Este punto deberá ser confirmado en el futuro.

El principal hallazgo del estudio bioquímico en la formación del complejo Gαq/PKCζ es la descripción de una región en Gαq homóloga a un dominio PB1. La mutación de este dominio interfiere con la asociación con PKCζ. Es interesante sugerir que el complejo Gαq/PKCζ sea el nexo entre la activación de GPCRs y procesos en los que se ha descrito

la importancia de dominios PB1. A través del mutante en la región pseudo-PB1 de Gαq hemos podido determinar el requerimiento estricto de la asociación con PKCζ para la activación de ERK5. Además, el hecho de que GRK2 sea un regulador de la asociación de PKCζ con Gαq y de la activación de ERK5 coincide con datos previos obtenidos para otros efectores de Gαq. Debido a que la deficiencia de asociación de Gαq con GRK2 potencia la vía de ERK5, sugerimos que, en aquellos contextos patológicos en los que los niveles de GRK2 se encuentran alterados, la vía de ERK5 podría estar hiperactivada con posibles implicaciones en dichas patologías.

Con respecto a los mecanismos de activación de PKCζ por GPCR, hemos descrito que no son dependientes de fosforilación pero que parecen involucrar la dimerización de PKCζ. Estos datos sugieren que PKCζ puede estar actuando de proteína andamio general de la vía, formando oligómeros que sirvan de plataforma de señalización para la activación de ERK5 por GPCRs. La presencia de Gαq activada y ERK5 en el mismo complejo molecular a través de PKCζ apoya dicha teoría. Así, proponemos un modelo de uniones secuenciales para explicar la activación de la vía. En este modelo, Gαq estaría activando PKCζ alostéricamente tal como se ha descrito para el resto de los efectores de Gαq y como previamente se ha demostrado que pueden actuar los dominios PB1 sobre PKCζ.

Por último, parece que el complejo Gαq/PKCζ juega un papel relevante en las funciones de "blebbing" y parada del ciclo celular dependientes de la activación del receptor muscarínico M3. De este modo, proponemos que la promoción de "blebbing" puede deberse a la reorganización del esqueleto de actina dependiente de ERK5 o a una posible activación de rhoA dependiente del complejo Gαq/PKCζ. Asimismo, la parada del ciclo celular puede suceder a través de JNK, tal como se he descrito recientemente. Por tanto, nuestros datos sugieren que el complejo Gαq/PKCζ podría ser responsable de esta activación. A su vez, hemos observado que el complejo Gαq/PKCζ participa en la promoción de apoptosis. Este resultado puede ser muy relevante en el sistema cardiovascular donde se ha visto que la sobreexpresión de Gαq puede conducir a fallo cardiaco. En este contexto, basándonos en datos preliminares, proponemos que la inhibición de la vía de Akt por Gαq puede ser dependiente de PKCζ. Asimismo, sugerimos que la función de la NADPH oxidasa, responsable de la producción de radicales libres de oxígeno y de la promoción de apoptosis es una diana potencial del eje de señalización Gαq/PKCζ. La descripción precisa de los eventos bioquímicos involucrados en las diversas funciones celulares del complejo Gαq/PKCζ deberá ser clarificada en futuros proyectos de investigación.

CONCLUSIONES

1. La vía de señalización Gαq/PKCζ/ERK5 es operativa en cardiomiocitos y fibroblastos, así como en corazones murinos *in vivo*.

2. La activación de ERK5 por receptores acoplados a Gαq es "Gαq-sesgada" y no requiere la internalización ni la fosforilación del receptor.

3. El complejo Gαq/PKCζ es detectado en células vivas y es altamente específico.

4. PKCζ interacciona con Gαq a través de su dominio PB1 tipo II. La lisina en posición 19 es un aminoácido clave para esta interacción. Por otra parte, Gαq interacciona con PKCζ a través de un dominio pseudo-PB1 type I localizado en la zona de unión a efector. Los ácidos glutámicos en posiciones 234 y 245 son cruciales en la interacción.

5. La formación de un complejo de señalización eficiente entre Gαq y PKCζ es esencial para la activación de ERK5 en respuesta a estimulación por GPCRs acoplados a Gαq.

6. GRK2 es un regulador negativo de la asociación entre Gαq y PKCζ. GRK2 también es un regulador negativo de la activación de ERK5 por GPCRs acoplados a Gαq.

7. PKCζ dimeriza de manera transitoria en respuesta a la estimulación de GPCRs acoplados a Gαq, y actúa como una proteína andamio entre Gαq activada y ERK5.

8. El complejo Gαq/PKCζ está involucrado en el proceso de "blebbing" y en la parada del ciclo celular inducidos por la activación del receptor muscarínico M3, y en la promoción de apoptosis.

IX. APPENDIX I

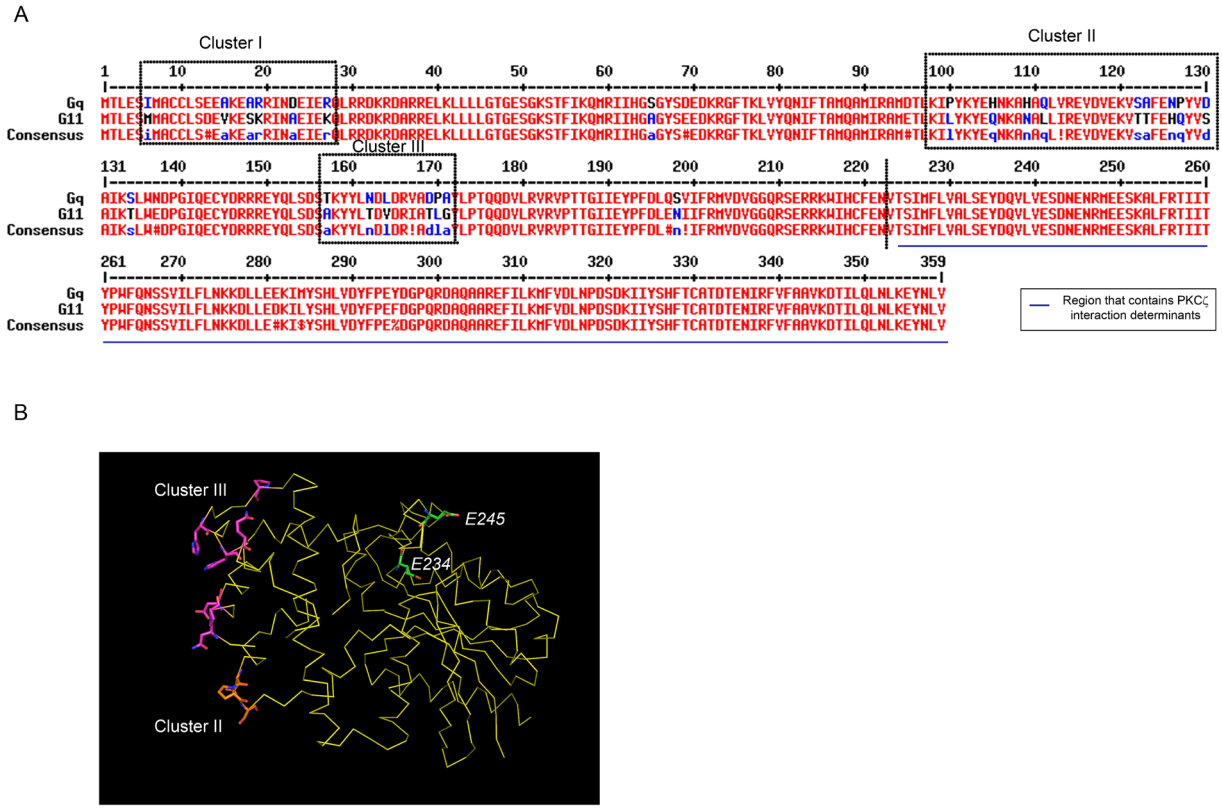

Figure 1. Primary sequence differences between Gαq and Gα11. (A) Sequence alignment between Gαq and Gα11 with highlighted variable clusters. Underlined in blue is the Gαq region determined to contain PKCζ-interaction derminants. (B) Cluster II and III, and aminoacids E234/E245 are marked in the crystal structure of Gαq (PDB accession number: 2BCJ).

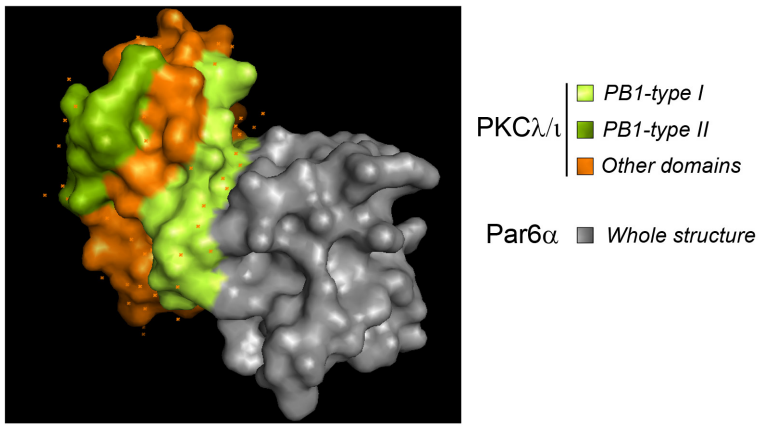

Figure 2. PB1typeII domain of PLCλ is free in the PLCλ-Par6 complex. Crystal structure of the complex between PLCλ (through its PB1 type I domain) and Par6 (through its PB1 type II domain) (PBD accession number: 1WMH).

X. APPENDIX II